ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

Founded by E. F. NORD

Edited by ALTON MEISTER

CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK

VOLUME 38

1973
AN INTERSCIENCE® PUBLICATION

JOHN WILEY & SONS
New York • London • Sydney • Toronto

ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

Volume 38

CONTRIBUTORS TO VOLUME 38

SIMON BLACK, *Laboratory of Biochemical Pharmacology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland*

ROSCOE O. BRADY, *Developmental and Metabolic Neurology Branch, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland*

SOO IL CHUNG, *Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland*

MAX A. EISENBERG, *Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York*

J. E. FOLK, *Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland*

MORRIS FRIEDKIN, *Department of Biology, University of California, San Diego, La Jolla, California*

DONALD J. MCNAMARA, *Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio*

VICTOR W. RODWELL, *Department of Biochemistry, Purdue University, Lafayette, Indiana*

DAVID J. SHAPIRO, *Department of Pharmacology, Stanford University Medical School, Stanford, California*

ROBERT T. SIMPSON, *Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland*

THRESSA C. STADTMAN, *Laboratory of Biochemistry, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland*

H. ZALKIN. *Department of Biochemistry, Purdue University, Lafayette, Indiana*

ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

Founded by E. F. NORD

Edited by ALTON MEISTER

CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK

VOLUME 38

1973
AN INTERSCIENCE® PUBLICATION

JOHN WILEY & SONS
New York • London • Sydney • Toronto

An Interscience® Publication

Library of Congress Catalog Card Number: 41-9213

ISBN 0-471-59173-4

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

CONTENTS

Anthranilate Synthetase
By H. Zalkin 1
Structure and Function of Chromatin
By Robert T. Simpson 41
Molecular and Catalytic Properties of Transglutaminases
By J. E. Folk and Soo Il Chung 109
A Theory on the Origin of Life
By Simon Black 193
Thymidylate Synthetase
By Morris Friedkin 235
Inborn Errors of Lipid Metabolism
By Roscoe O. Brady 293
Biotin: Biogenesis, Transport, and Their Regulation
By Max A. Eisenberg 317
Regulation of Hepatic 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase
By Victor W. Rodwell, Donald J. McNamara, and David J. Shapiro 373
Lysine Metabolism by Clostridia
By Thressa C. Stadtman 413
Author Index 449
Subject Index 473
Cumulative Index, Vols. 1-38 481

ANTHRANILATE SYNTHETASE

By H. ZALKIN, *Lafayette, Indiana*

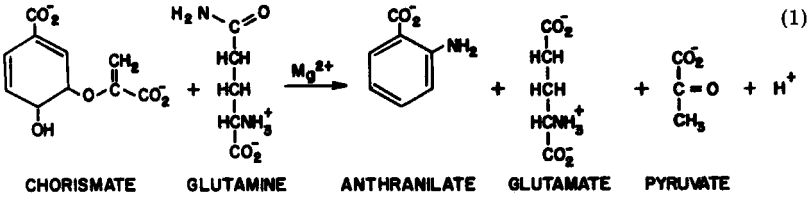
CONTENTS

I.	Introduction	1
II.	Aggregates of Anthranilate Synthetase	2
III.	Subunit Composition of Various Types of Anthranilate Synthetase	3
	A. Type I	3
	B. Type II	10
	C. Type III	13
IV.	Mechanism for Glutamine Utilization	15
V.	Anthranilate-5-Phosphoribosylpyrophosphate Phosphoribosyltransferase (PR Transferase) Associated with Type II Anthranilate Synthetase	19
VI.	On the Question of Intermediate Steps in the Conversion of Chorismate to Anthranilate	24
VII.	Tryptophan Inhibition	25
VIII.	Subunit Interactions and Cooperative Kinetics	27
IX.	Possible Physiological Function of Multienzyme Complexes	28
X.	Relationship to Other Glutamine Amidotransferases	29
XI.	Speculations on Evolutionary Relationships	32
XII.	Addendum	33
	Acknowledgment	36
	References	37

I. Introduction

Anthranilate synthetase catalyzes the first specific reaction for tryptophan synthesis in all microorganisms thus far studied (1) and perhaps also in plants (2). This reaction is shown in equation 1. Similar to other glutamine amidotransferases (3), NH_3 can replace glutamine, in which case the products of the reaction are anthranilate, pyruvate, and H_2O.

Anthranilate synthetase enzymes from all species so far examined are oligomeric proteins containing nonidentical subunits. The subunits are designated anthranilate synthetase Components I and II (4). Both subunits are required for glutamine-dependent



(1)

enzyme activity, but anthranilate synthetase Component I, by itself, catalyzes product formation using NH_3 as amino donor. Recent evidence, to be reviewed, indicates that anthranilate synthetase Component II provides the glutamine binding site.

Anthranilate synthetase enzymes from most species are subject to end product inhibition by tryptophan. Tryptophan binds to anthranilate synthetase Component I.

II. Aggregates of Anthranilate Synthetase

Multifunctional enzymes or enzyme aggregates have been detected in the tryptophan pathway of many organisms. However, in species of *Pseudomonas* (5), *Chromobacterium violaceum* (6), and *Bacillus subtilis** (7) aggregates or multifunctional enzymes are not found. Several patterns of association of anthranilate synthetase have been reported. These patterns are summarized in Table I. In bacteria, two types of anthranilate synthetase have been recognized (9). Type I anthranilate synthetases are oligomeric proteins not associated with other enzymes of the tryptophan biosynthetic pathway. Enzymes of type I have been isolated from *C. violaceum* (6), *B. subtilis* (10,11), species of *Pseudomonas* (12), and *Serratia marcescens* (9). Type II anthranilate synthetases are oligomeric proteins found in association with the second enzyme of the tryptophan biosynthetic pathway anthranilate - 5 - phosphoribosylpyrophosphate phosphoribosyltransferase (PR transferase). Enzymes of type II have been isolated and at least partially characterized from *Escherichia coli* (13,14), *Aerobacter aerogenes* (15,16), and *Salmonella typhimurium* (17,21).

* A contrary conclusion has been reported (8), but the most direct evidence argues against aggregate formation for tryptophan biosynthetic enzymes in *B. subtilis*.

A third type of anthranilate synthetase has been detected in yeast and fungi (22,23). In these organisms anthranilate synthetase is normally isolated in association with indole glycerol 3-phosphate synthetase or with indole glycerol 3-phosphate synthetase and *N*-(5′-phosphoribosyl)anthranilate isomerase activities. It is apparent from this summary that anthranilate synthetase may or may not be associated with other enzymes of the tryptophan pathway and that tryptophan synthetase is the only enzyme of the pathway not aggregated with anthranilate synthetase in any organism.

The summarized data on subunit composition and molecular weights in Table I are discussed in the next section.

III. Subunit Composition of the Various Types of Anthranilate Synthetase

In no case has the subunit composition of anthranilate synthetase from any organism been documented with unequivocal physiochemical evidence. Furthermore, in some instances deductions are made from studies with crude or partially purified enzymes. Nevertheless it appears that a compilation and analysis of presently available data may provide insights into structure-function and evolutionary relationships. For example, it appears that all anthranilate synthetase enzymes are oligomeric proteins containing nonidentical polypeptide chains. These protein chains are designated anthranilate synthetase Components I and II (4). Each component contributes specialized functions to the enzymatic reaction.

A. TYPE I

Anthranilate synthetase enzymes in this class are not aggregated to other proteins of the tryptophan pathway, according to gel filtration or sucrose gradient centrifugation analyses made on crude extracts. In addition other enzymes of the tryptophan pathway are removed upon partial or complete purification of anthranilate synthetase. It is of course possible that subtle physiologically important associations are destroyed upon cell disruption.

TABLE I

Patterns of Aggregation and Subunit Composition of Anthranilate Synthetase in Microorganisms[a]

Type	Organism	Activities aggregated to anthranilate synthetase	Subunit composition[b]	Molecular weight		Reference
				Component I	Component II	
1	*Bacillus subtilis*[c]	None	I_1II_1	80,000[d]	16,000	11
	Chromobacterium violaceum	None	—	—[e]	—	6
	Pseudomonas putida	None	I_1II_1	64,000	18,000	12
	Pseudomonas aeruginosa	None	I_1II_1	64,000	18,000	12
	Pseudomonas acidovorans	None	I_2II_2	71,000	18,000	12
	Pseudomonas testosteroni	None	I_2II_2	71,000	18,000	12
	Serratia marcescens	None	I_2II_2	60,000	21,000	9
	Salmonella typhimurium[f]	None	I_2II_2*	64,000	15,000–19,000	26
2	*Aerobacter aerogenes*	PR transferase	—	—	—	15, 16
	Escherichia coli	PR transferase	I_2II_2	60,000	60,000	13, 14
	Salmonella typhimurium	PR transferase	I_2II_2	64,000	63,000	21
3	*Aspergillus nidulans*	InGP synthetase and PRA isomerase	—	—	—	22
	Neurospora crassa	InGP synthetase and PRA isomerase	$I_2II_2III_4$[g]	40,000	30,000	23, 29
	Saccharomyces cerevisiae	InGP synthetase	—	—	—	22, 27

[a] Modified from Henderson (24).

[b] Anthranilate synthetase Components I and II are abbreviated I and II. Other abbreviations are PR transferase, anthrani-

late-5-phosphoribosylpyrophosphate phosphoribosyltransferase; InGP synthetase, indole glycerol 3-phosphate synthetase; PRA isomerase, *N*-(5′-phosphoribosyl) anthranilate isomerase.

[c] Anthranilate synthetase from *B. alvei* (25) may be analogous based on similarities of molecular weight.

[d] Calculated from the difference between the approximate molecular weight of the oligomeric enzyme and that of anthranilate synthetase Component II. This value and the subunit composition are highly provisional, as noted in the text.

[e] Subunit molecular weight was not estimated. A value of 80,000 to 95,000 was estimated for the intact enzyme.

[f] Native type II anthranilate synthetase that has been digested with trypsin. A fragment of II designated II* is associated with I.

[g] III designates PRA isomerase-InGP synthetase. The number of II subunits was not specified by the original authors (29) but has been tentatively indicated as 2 for this table (see text).

Anthranilate synthetase from crude extracts of *Serratia marcescens* was shown by Hutchinson and Belser (30) to separate from other tryptophan biosynthetic enzymes following sucrose gradient centrifugation. The enzyme was later purified to homogeneity (9,31). A molecular weight of approximately 140,000 was estimated by sucrose gradient centrifugation (9,31) while a value of approximately 150,000 was obtained by gel filtration (31). Gel electrophoresis in urea or sodium dodecyl sulfate revealed the presence of nonidentical polypeptide chains (9). A typical result is shown in Figure 1. Molecular weights of approximately 60,000 and 21,000 were estimated for the polypeptide chains by sodium dodecyl sulfate gel electrophoresis. In accord with the nomenclature introduced by Ito and Yanofsky (4) the large subunit was provisionally designated anthranilate synthetase Component I

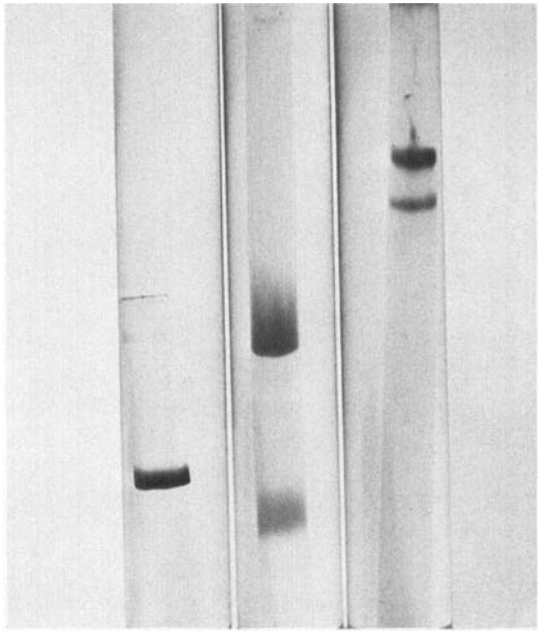


Fig. 1. Gel electrophoresis of anthranilate synthetase from *S. marcescens*. *Left*, homogeneous native enzyme; *middle*, 0.1% sodium dodecyl sulfate plus 0.1% mercaptoethanol; *right*, 8 *M* urea.

and the small subunit anthranilate synthetase Component II. According to this nomenclature unaggregated anthranilate synthetase Component I should contain sites for chorismate and NH_3 and therefore should catalyze anthranilate formation from these substrates. The unaggregated component I should be inactive with glutamine and reactivity with glutamine should be restored upon addition of anthranilate synthetase Component II. These criteria have yet to be fulfilled for the enzyme from *S. marcescens* since separation of the two types of subunits has not been achieved. However other evidence to be described justifies application of this nomenclature.

Evidence supporting a subunit composition of two polypeptide chains of each component (I_2II_2), as shown in Table I, was obtained from intramolecular cross-linking and affinity labeling experiments (9). Most of the eight species expected from random intramolecular crosslinking of a tetramer containing subunits of two sizes using dimethylsuberimidate (32) were detected by sodium dodecyl sulfate gel electrophoresis. Significantly, a protein of molecular weight approximately 40,000 was found following treatment with dimethylsuberimidate suggesting that the oligomeric enzyme might contain two chains of anthranilate synthetase Component II. Further evidence for two chains of anthranilate synthetase Component II was provided by affinity labeling with the glutamine analog 6-diazo-5-oxonorleucine (DON) and with iodoacetamide. Evidence to be presented in Section IV indicates that DON (and by analogy glutamine) bind to a sulfhydryl group of anthranilate synthetase Component II. Approximately 2 moles of DON and iodoacetamide were incorporated per mole of enzyme. Assuming one site per polypeptide chain, this result indicates two chains of anthranilate synthetase Component II per enzyme. Two chains of anthranilate synthetase Component I per enzyme molecule are expected based on a molecular weight of 140,000 to 150,000 for the oligomer. There is reasonable agreement between the sum of the approximate molecular weights of the polypeptide chains assuming a tetrameric composition of I_2II_2 (162,000) and the experimental determination of 140,000 to 150,000 for the oligomer.

Anthranilate synthetase enzymes from species of *Pseudomonas* were studied by Queener and Gunsalus (12,33). An oligomeric

enzyme of molecular weight approximately 65,000 to 73,000 was obtained from *Pseudomonas putida.* Nonidentical polypeptide chains of molecular weight approximately 64,000 and 18,000 corresponding to anthranilate synthetase Components I and II were purified to homogeneity. From this organism the subunits dissociate readily and it was demonstrated that anthranilate synthetase Component I catalyzes the reaction with chorismate and NH_3 as indicated.

$$\text{Chorismate} + NH_3 \xrightarrow{Mg^{2+}} \text{anthranilate} + \text{pyruvate} + H_2O \qquad (2)$$

No activity with glutamine was obtained. Upon addition of anthranilate synthetase Component II reactivity with glutamine was restored. It was concluded that upon interaction of subunits an oligomer of one chain of each component was formed (I_1II_1) as shown in Table I.

A study of anthranilate synthetase enzymes (12) from *Pseudomonas aeruginosa, Pseudomonas acidovorans,* and *Pseudomonas testosteroni* showed that the enzymes could be divided into two groups: *putida-aeruginosa* (p-a class) and *acidovorans-testosteroni* (c-t class). Enzyme from *Pseudomonas stutzeri* resembled the p-a class and *Pseudomonas multivorans* the c-t class. The c-t class anthranilate synthetase Components I and II separated with difficulty, and the aggregate appeared to be larger than the more freely dissociable p-a complexes. Sucrose gradient centrifugation analyses of crude extracts suggested a molecular weight of approximately 155,000 for the oligomeric enzymes from the c-t class. Partially purified anthranilate synthetase Component I from this class was of molecular weight approximately 71,000 while the anthranilate synthetase Component II was similar in size to that from the p-a class, molecular weight approximately 18,000. On the basis of size relationships a composition of I_2II_2 was suggested for the c-t class anthranilate synthetase. Aggregation of isolated components to form oligomer, as measured by appearance of glutamine-dependent anthranilate synthetase activity, was most efficient using enzyme components of the same class yet interclass complementation was obtained. Thus various hybrid enzymes were presumably formed. These relationships are summarized in Table I.

An oligomeric anthranilate synthetase of molecular weight approximately 96,000 containing nonidentical subunits was detected in *Bacillus subtilis* (10,11). Anthranilate synthetase Component II (dubbed "subunit-X") of molecular weight approximately 16,000 was isolated and partially purified but free component I was not obtained. The results were complicated by nonlinearity between enzyme concentration and velocity of glutamine-dependent anthranilate synthetase under conditions of excess component II. Anthranilate synthetase Component II stimulated crude or partially purified glutamine-dependent enzyme activity of the aggregate and restored glutamine-dependent activity to the aggregate from a mutant strain containing inactive component II. These results suggest that *(a)* the aggregate of molecular weight approximately 96,000 may not have been fully saturated with anthranilate synthetase Component II and *(b)* active component II exchanged with the inactive component in the aggregate from the mutant strain. Anthranilate synthetase from *B. subtilis* may be an oligomer of composition I_1II_1 as shown in Table I, but this speculation by the reviewer is tenuous and a more confident description of the enzyme must await further experimentation.

A study of the mutant strain of *B. subtilis,* which contains defective anthranilate synthetase Component II, has provided several interesting additional results (11). *Trp*X which encodes anthranilate synthetase Component II was unlinked by DNA transformation or transduction analyses to the tryptophan gene cluster, yet synthesis of component II was regulated coordinately with the tryptophan synthetase B protein under most conditions (11). Since *trp*X mutants lacking anthranilate synthetase Component II were prototrophic for tryptophan, *in vivo* utilization of NH_3 was suggested. In addition a role was suggested for anthranilate synthetase Component II in the synthesis of 4-aminobenzoate. This is discussed in Section X.

Molecular weight estimations of approximately 80,000 to 95,000 were made for relatively crude preparations of anthranilate synthetase from *Bacillus alvei* (25) and *Chromobacterium violaceum* (6). The enzyme from *C. violaceum* was clearly separated from other enzymes of tryptophan biosynthesis but such information has not been verified for *B. alvei* anthranilate

synthetase. Although no data on subunit composition are yet available, a composition of I_1II_1 could be accommodated by the estimated molecular weights and by analogy to other anthranilate synthetase enzymes described in this section.

Trypsin-treated anthranilate synthetase from *S. typhimurium* is a type I enzyme prepared *in vitro* from the native type II oligomer and is discussed in the next section. As noted in Table I, there is little variation in the approximate molecular weights for type I anthranilate synthetase Components I and II.

B. TYPE II

Anthranilate synthetase enzymes designated type II are aggregated to the second enzyme of the tryptophan pathway, PR transferase. Anthranilate synthetase–PR transferase aggregates have been characterized to varying extents from *E. coli*, *A. aerogenes*, and *S. typhimurium*.

The report by Ito and Yanofsky (4) on "The Nature of the Anthranilic Acid Synthetase Complex of *Escherichia coli*" provided the starting point for all subsequent investigations on the subunit composition of this enzyme. Anthranilate synthetase in extracts of *E. coli* was characterized as an oligomer containing nonidentical subunits. The subunits are the products of the *E. coli trp*E and *trp*D genes. The *trp*E gene product obtained from a *trp*D nonsense mutant was designated anthranilate synthetase Component I. Anthranilate synthetase Component I had a sedimentation coefficient of approximately 4.3 S and lacked glutamine-dependent enzyme activity. It was reported that this protein was "activated" by NH_4^+ ions. It is now recognized that anthranilate synthetase Component I utilizes NH_3 as a substrate together with chorismate for synthesis of anthranilate and pyruvate. The gene product of *trp*D, PR transferase ($s_{20,w}$ approximately 4.4 S) was obtained from a *trp*E nonsense mutant and was required for glutamine-dependent anthranilate synthetase activity in association with anthranilate synthetase Component I. PR transferase was thus designated anthranilate synthetase Component II. Anthranilate synthetase-PR transferase obtained from wild type cells or by mixing extracts containing each of the two components had a sedimentation constant of approximately 7.5 S. These results established that nonidentical

subunits are required for glutamine-dependent anthranilate synthetase activity in *E. coli.*

The molecular weight of essentially homogeneous *E. coli* anthranilate synthetase Component I was reported to be approximately 60,000 to 63,000 (34). The molecular weight of *E. coli* anthranilate synthetase Component II has not been reported. Ito and Yanofsky have stated (13) that preliminary investigations on the molecular weight of the *E. coli* anthranilate synthetase complex, and its subunit composition, give values of 260,000 ± 20,000 and two subunits of each component in the complex.

Anthranilate synthetase PR transferase has been highly purified from *A. aerogenes* (15,16). A sedimentation constant ($s_{20,w}$) of 8.1 S for the aggregate and 4.1 S for anthranilate synthetase Component II was reported (16). Information on the subunit composition of anthranilate synthetase–PR transferase from *A. aerogenes* is not available.

The evidence pointing to a subunit composition of I_2II_2 for anthranilate synthetase–PR transferase from *S. typhimurium* is presently the most complete for any anthranilate synthetase enzyme. The evidence is based on a consideration of the molecular weights of the isolated components and the intact aggregate, stoichiometry of ligand binding, and desitometric analysis following electrophoretic separation and staining of subunits.

On the basis of *in vitro* complementation experiments similar to those performed with extracts of *E. coli* (4), Bauerle and Margolin (17) deduced that anthranilate synthetase from *S. typhimurium* was an oligomer containing nonidentical subunits: anthranilate synthetase Component I catalyzed product formation from chorismate and NH_3 and anthranilate synthetase Component II conferred glutamine reactivity upon component I. Component II by itself also contained PR transferase activity.

Homogeneous anthranilate synthetase Component I was characterized as a single polypeptide chain ($s^\circ_{20,w}$ = 3.8 S) of molecular weight 64,000 (35). This conclusion was based on finding similar molecular weights for the native and denatured component I using gel filtration (36), sedimentation equilibrium centrifugation in the presence and absence of 8 *M* urea or 6 *M* guanidine hydrochloride (35) and sodium dodecyl sulfate gel electrophoresis. Anthranilate synthetase Component II (PR transferase)

was reported to be a polypeptide chain of molecular weight approximately 63,000 but suggestions of aggregation were noted (19). Recent genetic studies also indicate that PR transferase is a single polypeptide chain (37). Electrophoresis of anthranilate synthetase–PR transferase in urea and sodium dodecyl sulfate confirms the presence of nonidentical subunits of similar size. Disc gel electrophoresis in 8 *M* urea reveals two protein bands corresponding to the isolated components (20,21). Sodium dodecyl sulfate gel electrophoresis, on the other hand, yields a single protein band of molecular weight approximately 62,000 (21). It appears unlikely that protein constituents other than anthranilate synthetase Components I and II could be contained in the aggregate.

The relative weight contributions of anthranilate synthetase Components I and II to the aggregate were estimated by integration of densitometer tracings of gels that were stained with Coomassie blue. The relative weight ratio of anthranilate synthetase Component I to Component II was 1.16. Since the subunits have similar molecular weights, equal weight contributions indicate equal numbers of polypeptide chains. A major assumption for these calculations is identical staining of subunits. Although there are elements of uncertainty in this assumption (38), similar analyses have provided well accepted conclusions about the subunit composition of *E. coli* RNA polymerase (39) and *E. coli* aspartate transcarbamylase (40). Supporting evidence for equal numbers of subunits was obtained from measurements of ligand binding. Binding of 1.9 moles of chorismate or of 1.8 moles of tryptophan per mole of enzyme was detected by equilibrium dialysis measurements (21). Chorismate and the feedback inhibitor tryptophan each bind to anthranilate synthetase Component I. Binding of approximately 2 moles of the glutamine analog DON per mole enzyme was shown to be due to covalent attachment to anthranilate synthetase Component II (20). Assuming one site per chain these results indicate two chains each of anthranilate synthetase Components I and II per enzyme molecule.

Molecular weight determinations also are compatible with a composition of I_2II_2 for the *Salmonella* enzyme. The best estimation of molecular weight for the aggregate is 280,000 determined by sedimentation equilibrium centrifugation (21). Other values

of approximately 261,000 to 290,000 were obtained by sucrose gradient centrifugation (18,26) and gel filtration (41). These molecular weight determinations are in reasonable agreement with the value of 254,000 calculated for I_2II_2 ($2 \times 64{,}000 + 2 \times 63{,}000$).

Of interest is a reported molecular weight of 137,000 for anthranilate synthetase from *S. typhimurium* (42). The discrepancy between this value and that of 280,000 was resolved when it was found that during purification a treatment with pancreatic lipase which is often contaminated with proteolytic enzymes caused digestion of the native enzyme (26). The decreased size following digestion of the aggregate with pancreatic lipase or trypsin resulted from digestion of anthranilate synthetase Component II from molecular weight approximately 63,000 to approximately 15,000 to 19,000. Loss of PR transferase activity occurred concomitantly. Anthranilate synthetase Component I was unaltered. It therefore follows that a component II fragment of molecular weight 15,000 to 19,000 can interact with component I and allow glutamine reactivity. It appears that the component II fragment from *S. typhimurium* and the component II chains of similar molecular weight from type I anthranilate synthetase enzymes function as a glutamine binding protein (Section IV). Trypsin-digested anthranilate synthetase from *S. typhimurium* has been included in Table I as a type I enzyme for comparison with those that occur *in vivo*.

Although characterization of the subunit composition has progressed furthest with anthranilate synthetase from *S. typhimurium* the enzymes from *E. coli* and *A. aerogenes* may be similar. *In vitro* complementation experiments (43) indicate facile formation of hybrid anthranilate synthetase aggregates using mixtures of subunits from the three organisms.

C. TYPE III

Anthranilate synthetase enzymes in this category are aggregated to either *N*-(5′-phosphoribosyl)anthranilate isomerase and indole glycerol 3-phosphate synthetase or to just the latter. It appears that such aggregates are found mainly in fungi and yeast although apparently not all fungi have multifunctional aggregates of anthranilate synthetase (22). The basic association appears to

be between anthranilate synthetase and indole glycerol 3-phosphate synthetase. If *N*-(5′-phosphoribosyl)anthranilate isomerase is associated with the latter to form a bifunctional enzyme, both activities are aggregated to anthranilate synthetase as in *Neurospora crassa,* for example. If, however, *N*-(5′-phosphoribosyl)anthranilate isomerase is a distinct gene product, as in *Saccharomyces cerevisiae,* it is not included in the anthranilate synthetase-indole glycerol 3-phosphate synthetase aggregate.

Purification to homogeneity and characterization of the anthranilate synthetase-*N*-(5′phosphoribosyl)anthranilate isomerase-indole 3-glycerol phosphate synthetase aggregate from *N. crassa* was reported by Gaertner and DeMoss (23). It was concluded that the isolated aggregate of molecular weight 240,000 ($s^{\circ}_{20,w}$ = 10.3 S) had a composition of I_2III_4 where I is anthranilate synthetase and III is *N*-(5′-phosphoribosyl)anthranilate isomerase-indole glycerol 3-phosphate synthetase.* Subunits of molecular weight 40,000 were detected by sedimentation equilibrium centrifugation in denaturing solvents. The native aggregate was dissociated into two fragments by addition of a 40-fold molar excess of *p*-mercuribenzoate or *o*-iodosobenzoate. One fragment, approximately 7.4 S (estimated molecular weight, 160,000) contained *N*-(5′-phosphoribosyl)anthranilate isomerase and indole glycerol 3-phosphate synthetase activity but not anthranilate synthetase activity. This fragment is designated III_4. The other fragment (I_2) approximately 4.4 S (estimated molecular weight, 80,000) was catalytically inactive. Fraction I_2 acquired minimal anthranilate synthetase activity following treatment with dithiothreitol and also appeared to be tetrameric according to sucrose gradient centrifugation analysis. It was concluded that I_2 may dimerize under certain conditions. It is likely that I_2 corresponds to dimeric anthranilate synthetase Component I.

In a recent abstract (28) and in the more complete report (29) it was stated for the first time that I_2III_4 has anthranilate synthetase activity with NH_3 but not with glutamine. This property identified I as anthranilate synthetase Component I. A previously unrecognized subunit of molecular weight approximately

* The authors' original abbreviations for designating the subunits of this enzyme (23,28,29) have been changed to conform with other enzymes in Table I.

30,000 was required to restore glutamine-dependent anthranilate synthetase activity to homogeneous I_2 or to I_2III_4 under appropriate conditions. Reconstitution of glutamine-dependent enzyme activity identifies the subunit of molecular weight approximately 30,000 as anthranilate synthetase Component II. Anthranilate synthetase Component II was isolated free or in association with the freshly prepared native enzyme of molecular weight approximately 300,000 or with the *N*-(5′-phosphoribosyl)anthranilate isomerase-indole glycerol 3-phosphate synthetase subunit (molecular weight approximately 200,000). Dissociation of component II from the latter two forms occurred readily. Either free component II or component II in association with *N*-(5′-phosphoribosyl)anthranilate isomerase-indole glycerol 3-phosphate synthetase interacted with anthranilate synthetase Component I to generate glutamine-dependent activity. Based on an estimated molecular weight of 300,000 for the glutamine-dependent enzyme and 240,000 for the strictly NH_3-dependent anthranilate synthetase complex and the 1:1 stoichiometry for components I and II in other organisms it appears that the native complex may contain two subunits of anthranilate synthetase Component II. A composition of $I_2II_2III_4$ is thus suggested in Table I, although the original authors (29) were careful to exercise restraint and not speculate on the number of component II subunits.

From this compilation (Table I) it is apparent that anthranilate synthetase enzymes from all species thus far examined are oligomers containing nonidentical subunits. One subunit, anthranilate synthetase Component I, is required for enzymatic catalysis with NH_3 and the second subunit, anthranilate synthetase Component II, allows utilization of glutamine.

IV. Mechanism of Glutamine Utilization

Studies with the glutamine analog 6-diazo-5-oxonorleucine (DON) have helped elucidate general features of the anthranilate synthetase reaction mechanism. Glutamine analogs, particularly DON, *o*-diazoacetyl-L-serine (azaserine), 2-amino-4-oxo-5-chloropentanoic acid, and L-2-amino-3-ureidopropionic acid (albizziin), inactivate the glutamine-dependent activity of several glutamine amidotransferases (3,44–52) while having little or no

effect upon the NH_3-dependent activity of many of these enzymes. Similarly the differential inhibitory or inactivating effect of DON had been noted with anthranilate synthetase (42,53).

A detailed study of the reaction of DON with anthranilate synthetase from *S. typhimurium* was conducted by Nagano et al. (20). DON was shown to inhibit glutamine-dependent anthranilate synthetase activity and also NH_3-dependent activity of anthranilate synthetase-PR transferase and anthranilate synthetase Component I. In each case inhibition was competitive with glutamine or NH_3. Inhibition of NH_3-dependent anthranilate synthetase activity by DON was ascribed to its action as a weak NH_3 analog. Of more importance, however, was *inactivation* of glutamine-dependent anthranilate synthetase activity of anthranilate synthetase-PR transferase by DON. Inactivation was irreversible. Activity was not regained following a hundredfold dilution, dialysis, or gel filtration. NH_3-dependent anthranilate synthetase activity of anthranilate synthetase–PR transferase was largely unaffected while the activity of anthranilate synthetase Component I was totally unaffected. Glutamine provided substantial protection against inactivation by DON. These results indicate that covalent attachment of DON to the glutamine site prevents binding or reaction of glutamine but not NH_3 and thus provide evidence for distinct sites for these two ligands. Anthranilate synthetase Component I lacks a functional glutamine binding site.

The rate of inactivation by low concentrations of DON was stimulated 25-fold or more by chorismate suggesting ordered binding of first chorismate and then DON, or by analogy glutamine. Mg^{2+}, which is required for enzyme activity, had a relatively small effect on the reaction with DON, suggesting that it is not required for binding of chorismate, DON, or glutamine. Tryptophan, on the other hand, completely prevented inactivation of glutamine-dependent anthranilate synthetase activity. This result indicates that the action of tryptophan as a feedback inhibitor is at an early step in the mechanism. Tryptophan could inhibit anthranilate synthetase activity by preventing binding of the first substrate, chorismate, or by preventing the putative conformational change required for glutamine to bind. Evidence for the former possibility is in Section VII. Analogs of tryptophan

decreased the rate of inactivation by DON in relation to their effectiveness as feedback inhibitors.

The conclusion that glutamine and NH_3 bind to distinct sites was verified when anthranilate synthetase and PR transferase subunits of enzyme labeled with ^{14}C-DON were separated by gel electrophoresis in 8 *M* urea. DON was attached to the PR transferase subunit; thus not only are the two sites distinct but they are on separate polypeptide chains. By a variety of techniques it was shown that each PR transferase chain contained approximately one site for DON and each molecule of anthranilate synthetase-PR transferase approximately two DON sites. Therefore anthranilate synthetase–PR transferase should contain two PR transferase subunits (excluding the unlikely possibility of "half site" reactivity (54). It was also shown that, following inactivation of the enzyme with DON, two cysteine groups per molecule were unavailable for tritation with 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) indicating alkylation of cysteine residues.

Binding of glutamine to anthranilate synthetase Component II and chorismate to Component I implies transfer of NH_3 between subunits. Khedouri et al. (49) had earlier suggested a mechanism for *E. coli* carbamyl phosphate synthetase invoking transfer of the amide of glutamine to an NH_3 site. A glutaminase activity that could reflect the transfer mechanism was detected with anthranilate synthetase–PR transferase. Glutaminase was assayed in the absence of overall synthesis of anthranilate. Glutaminase activity was stimulated by chorismate and inhibited by tryptophan. The effects of chorismate and tryptophan are explained as for the reaction with DON. *(a)* The requirement for chorismate is a reflection of ordered binding of first chorismate and then glutamine. *(b)* Tryptophan inhibits binding of either chorismate or glutamine. The rate of glutaminase was similar to the overall rate of glutamine-dependent anthranilate synthetase and was only detected in anthranilate synthetase–PR transferase and not in either of the unaggregated components. Isolation of γ-glutamyl hydroxamate (55,56) following incubation of the enzyme with chorismate, glutamine, and hydroxylamine provides evidence for a γ-glutamyl-enzyme thioester. Hydroxamate formation, dependent upon chorismate and indicative of glutaminase,

has been detected with anthranilate synthetase from *E. coli* (57) and *P. putida* (33).

The scheme shown in Figure 2 summarizes our present view of the anthranilate synthetase mechanism. The scheme shows ordered binding of first chorismate to anthranilate synthetase Component I and then binding of glutamine to a cysteine residue of anthranilate synthetase Component II. Amide transfer from glutamine on component II to the NH_3 site on component I is followed by anthranilate formation on component I. Hydrolysis of the γ-glutamyl-thioester on anthranilate synthetase Component II gives release of glutamate. The last step shows release of anthranilate and pyruvate. There is no evidence for the order of product release. It is suggested that amide transfer is to the site on component I that utilizes NH_3 from solution.

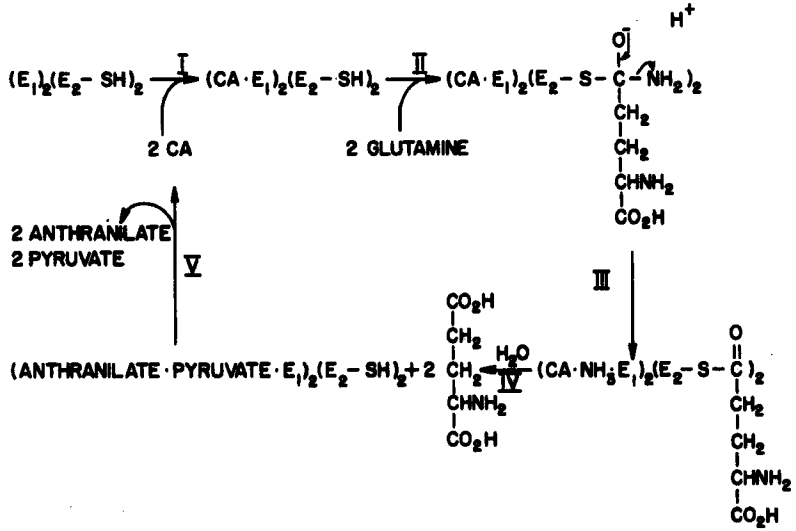


Fig. 2. Minimal hypothetical reaction mechanism for glutamine-dependent anthranilate synthetase activity of anthranilate syntehtase-PR transferase. E_1, E_2, and CA are anthranilate synthetase Component I, PR transferase (anthranilate synthetase Component II), and chorismate, respectively. Step I shows reaction of chorismate with component I of the aggregate. Step II shows reaction of glutamine with component II of $(CA \cdot E_1)_2(E_2\text{-}SH)_2$. Step III shows amide transfer from E_2 • glutamine of $(CA \cdot E_1)_2(E_2\text{-S-glutamyl})_2$ to component I. Step IV shows synthesis of anthranilate and pyruvate by component I and release of glutamate from component II. Step V shows release of anthranilate and pyruvate from component I. See the text for further description. From reference 20.

Experiments on the relationships between the two functions of PR transferase, glutamine binding, and phosphoribosylanthranilate synthesis, are reviewed in Sections III.B and V. It appears that the *S. typhimurium trp*B gene product [or the *E. coli trp*D gene product (37)] is bifunctional and contains an NH_2-terminal segment for glutamine binding and a larger segment for PR transferase activity.

Type I anthranilate synthetase enzymes may utilize a mechanism similar to that described in Figure 2. Such enzymes have been studied from *P. putida* (33) and *S. marcesens* (9). Queener (33) has independently proposed a mechanism for *P. putida* anthranilate synthetase essentially similar to that shown in Figure 2. According to Queener the amide of glutamine may be transferred directly to chorismate. In the mechanism proposed by Nagano et al. the amide of glutamine is transferred to the NH_3 site on anthranilate synthetase Component I.

Recent results with anthranilate synthetase from *B. subtilis* (11) are difficult to accommodate with the proposed mechanism. It has been reported that the glutamine-dependent activity for this enzyme was up to 6.5 times faster than the NH_3-dependent activity (Table II in ref. 11). Furthermore, a nonlinear relationship between enzyme concentration and velocity of glutamine-dependent anthramilate synthetase was obtained under conditions of excess component II. These results suggest additional complexities in the reaction mechanism, which are not presently understood.

V. Anthranilate-5-Phosphoribosylpyrophosphate Phosphoribosyltransferase (PR Transferase) Associated with Type II Anthranilate Synthetase

A brief review of the PR transferase that is associated with anthranilate synthetase Type II seems justified, since the polypeptide chain containing PR transferase is required for glutamine-dependent anthranilate synthetase activity. The main questions to consider are *(a)* the relationship between the glutamine binding function and PR transferase activity, *(b)* possible role of PR transferase in oligomer formation, *(c)* the mechanism for inhibition of PR transferase by tryptophan, and *(d)* effect of anthranilate synthetase Component I on PR transferase.

PR transferase from *E. coli* (37) and *S. typhimurium* (21,26) is a single polypeptide chain containing a glutamine binding site for anthranilate synthetase activity and a PR transferase catalytic site. Treatment of anthranilate synthetase-PR transferase with crude pancreatic lipase or with trypsin digests PR transferase from molecular weight approximately 63,000 to a fragment of about 15,000 to 19,000 with resultant loss of PR transferase activity (26). Glutamine-dependent anthranilate synthetase activity is unaltered indicating that the component II fragment is functional for glutamine binding and amide transfer. The component II fragment has also been isolated following proteolytic digestion of unaggregated PR transferase (58,59). In neither instance was PR transferase activity recovered. The component II fragment generated *in vitro* by enzymatic digestion is approximately 24 to 38% of the entire protein chain of molecular weight 63,000. Analysis of *E. coli trp*D mutants indicates that the operator proximal 25% of the gene is required for transcription and translation of a functional anthranilate synthetase Component II fragment (37). A value of approximately 40% is estimated for the *S. typhimurium trp*B gene (59). This peptide fragment must therefore correspond to the amino-terminal portion of the protein. The simplest interpretation of these results is that the product of the *trp*B gene in *S. typhimurium* and *trp*D in *E. coli* is bifunctional and contains a glutamine binding site and a PR transferase catalytic site (Fig. 3). It would be necessary to isolate a fragment possessing PR transferase activity before it could be concluded that each function is completely independent of the other.

Grieshaber and Bauerle (59) have conducted a genetic and biochemical analysis of the *S. typhimurium trp*B gene product. The size of the component II fragment resulting from a nonsense mutation in region 2 of the *trp*B gene (Figure 3) was related to the availability of anthranilate synthetase Component I *in vivo*. In strains containing component I, the aggregated component II fragment was of uniform size (molecular weight approximately 24,000) regardless of the site of the nonsense mutation in region 2. *In vivo* proteolysis to this minimal component II core fragment is therefore indicated. In strains not containing component I protein, nonsense mutations in region 2 produced component II

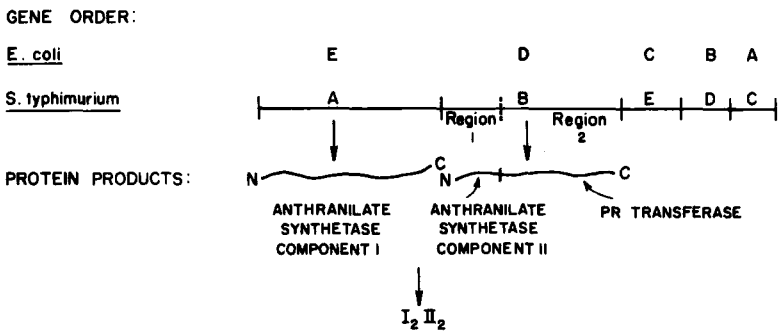


Fig. 3. Schematic represensation of gene-protein relationships for part of the *trp* operon in *E. coli* and *S. typhimurium*. Genes are drawn to arbitrary lengths. The product of *S. typhimurium trp*A is anthranilate synthetase Component I, and the product of *trp*B is shown as a bifunctional polypeptide chain containing anthranilate synthetase Component II and PR transferase activities. N and C refer to the amino and carboxyl termini. Aggregation to the tetrameric oligomer is arbitrarily shown to occur after synthesis of the polypeptide chains. *I* designates anthranilate synthetase Component I, *II* the bifunctional anthranilate synthetase Component II. Region 1 corresponds to anthranilate synthetase Component II and region 2 to PR transferase.

fragments of varying size. These fragments of heterogeneous size were susceptible to digestion *in vitro* using trypsin to yield component II core fragment of molecular weight approximately 24,000. The kinetics for treatment of native PR transferase with trypsin suggest sequential digestion to produce fragments of decreasing size: 62,000 → 50,000 → 35,000 → 24,000. It is apparent that proteolysis is complex and does not involve a single susceptible site connecting the two putative peptide functions of the bifunctional protein. The use of different empirical techniques for molecular weight estimation of the component II core fragment accounts for differences in reported values (26,59).

Genetic analyses indicate that a functional amino-terminal anthranilate synthetase Component II segment is not obligatory for PR transferase activity *in vivo*. Mutations in the operator proximal end of *trp*B in *S. typhimurium* [orginally designated "unusual," more recently called region 1 (59)] lead to a decrease or loss of glutamine-dependent anthranilate synthetase activity (17). Tryptophan auxotrophy or bradytrophy results from defective anthranilate synthetase Component II. Since these mutants

grow on anthranilate, it appears likely that they contain functional PR transferase. The *trp*B region 1 mutants contain anthranilate synthetase Component I. *E. coli* strains with chain terminating mutations in the operator proximal end of *trp*D corresponding to the region coding for anthranilate synthetase Component II also grow on anthranilate and exhibit low but detectable PR transferase activity *in vitro* (37). Such strains contained anthranilate synthetase Component I but not glutamine-dependent enzyme activity. It was suggested that a proper initial segment of the *E. coli trp*D polypeptide (anthranilate synthetase Component II) was not present. Such strains may therefore contain an active fragment of PR transferase.

In addition to providing the glutamine binding site, anthranilate synthetase Component II is required for aggregate formation. Bauerle and Margolin (17) noted that anthranilate synthetase Component I in region 1 nonsense mutants of *trp*B was monomeric and was not complexed with residual PR transferase. In this laboratory, it has been shown that anthranilate synthetase Component I from *S. typhimurium* is monomeric under usual conditions in the crude or purified states (35). Anthranilate synthetase–PR transferase is, on the other hand, tetrameric (21). Following enzymatic digestion of oligomeric PR transferase the enzyme remains tetrameric (26). Crude glutamine-dependent anthranilate synthetase from *S. typhimurium trp*B region 2 nonsense mutants is similar in size to the trypsin-digested enzyme (Nagano, unpublished; ref. 59). It therefore follows that the component II fragment of molecular weight 15,000 to 19,000, not the entire PR transferase protein, is required for oligomer formation.

Tryptophan inhibition of PR transferase activity of anthranilate synthetase-PR transferase is only partial (13,15,17,19) indicating that the tryptophan regulatory site and the catalytic site are distinct. Further evidence for this conclusion is that maximal tryptophan inhibition of *S. typhimurium* PR transferase activity required saturating concentrations of both substrates (19,60). There is presently no explanation for this result although other examples were cited (19) for inhibition dependent on saturating levels of substrates. More recently other cases have been reported for enhanced binding of regulatory effectors in the presence

of substrate (61) or products (62). While it is clear that the tryptophan regulatory site must be distinct from the PR transferase catalytic site, it is unclear whether the regulatory site is even on the PR transferase subunit. Some evidence supports the view that tryptophan bound to anthranilate synthetase Component I causes partial inhibition of PR transferase activity although other observations are inconsistent with this view. The evidence favoring the hypothesis that the only tryptophan binding site is on anthranilate synthetase Component I is as follows. *(a)* Unaggregated PR transferase was only very weakly (19) or not at all (13,17) inhibited by tryptophan. *(b)* No binding of tryptophan to PR transferase of anthranilate synthetase–PR transferase was detected under conditions known to give inhibition (21). *(c)* With anthranilate synthetase Component I chorismate and tryptophan exhibit competitive kinetics (35). Chorismate likewise antagonized tryptophan inhibition of PR transferase activity, suggesting that exclusion of tryptophan binding to component I prevents inhibition of PR transferase (19). Furthermore, similar cooperativity for chorismate was obtained for saturating anthranilate synthetase and antagonizing tryptophan inhibition of PR transferase activity. *(d)* PR transferase enzymes are not inhibited by tryptophan in organisms where aggregation to anthranilate synthetase does not occur. Therefore tryptophan inhibition of PR transferase activity in type II enzymes probably does not exert an essential function.

Evidence difficult to reconcile with a single tryptophan site on anthranilate synthetase Component I includes the following. *(a)* Mg^{2+} reversed inhibition of anthranilate synthetase by tryptophan but was required for inhibition of PR transferase (19). *(b)* Very different pH profiles were obtained for tryptophan inhibition of anthranilate synthetase and PR transferase activities. *(c)* Chorismate did not antagonize the weak tryptophan inhibition of unaggregated PR transferase. *(d)* Tryptophan inhibition of PR transferase aggregated to feedback-insensitive anthranilate synthetase was similar to that obtained for PR transferase aggregated to wild type anthranilate synthetase (13). For the latter, the authors were careful to point out that tryptophan may bind to feedback-insensitive anthranilate synthetase Component I and inhibit PR transferase although unable to inhibit anthranilate

synthetase. In this reviewer's opinion it is not yet clear whether or not PR transferase has a tryptophan binding site.

It is clear that association of PR transferase with anthranilate synthetase Component I influences the properties of both proteins. In addition to providing increased sensitivity to tryptophan inhibition, aggregation also increases the heat stability of PR transferase activity (13,17).

VI. On the Question of Intermediate Steps in the Conversion of Chorismate to Anthranilate

The complexity in the conversion of chorismate into anthranilate has invited numerous speculations about the course of the reaction (63–66). However chemical intermediates have not been identified. The only partial reaction detected is glutaminase (20,57) which appears to be associated with the mechanism of glutamine utilization (Section IV). Formation of α-carboxy-α-*N*-dimethylnitrone by reaction of *N*-methyl hydroxylamine with the enolpyruvyl group of chorismate or with pyruvate may (55) or may not (56) be enzymatic and is of unknown significance. Based on low incorporation of ^3H from $^3H[H_2O]$ into the product pyruvate, it was concluded that the proton from C-2 of chorismate was transferred to the leaving enolpyruvate group (36). This was later proven to be incorrect (67,68). The third hydrogen atom of the methyl group of pyruvate originates from water protons.

Inactivation of *S. typhimurium* anthranilate synthetase Component I by bromopyruvate (36) may result from alkylation of an amino acid residue in close proximity to the site for chorismate. Chorismate and tryptophan reduced the rate of inactivation by bromopyruvate. The possibility was considered that bromopyruvate may alkylate a basic group required for abstraction of the C-2 proton from the proposed intermediate 2,3-dihydro-3-enolpyruvylanthranilate (Fig. 4). Anthranilate synthetase component I from *P. putida* was not inactivated by bromopyruvate (33).

In the absence of firm evidence it is best to retain the simple mechanism originally proposed by Levin and Sprinson (64) and Srinivasan (65) as a working hypothesis in preference to more

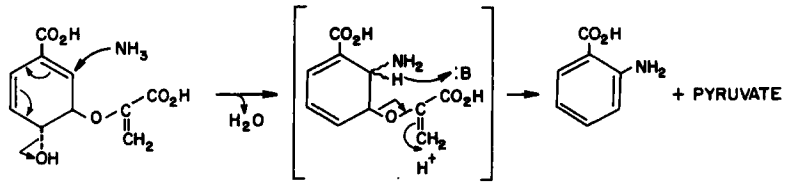


Fig. 4. Hypothetical mechanism of the anthranilate synthetase reaction modified from Levin and Sprinson (64) and Srinivasan (65). NH_3, exogenous or arising from glutamine, is shown to react with chorismate to give the postulated intermediate 2,3-dihydro-3-enolpyruvylanthranilate. The sterochemistry of the amino group and hydrogen atom is not specified. A basic group, :B, may abstract the hydrogen atom on C-2 and initiate displacement of enolpyruvate.

complicated alternatives (63,66). The former is slightly modified in Figure 4 to account for recent information on utilization of NH_3, generation of NH_3 from glutamine, and the possible function of a basic group in abstraction of the ring proton.

VII. Tryptophan Inhibition

Anthranilate synthetase Component I from *E. coli* (34) and *S. typhimurium* (36) is subject to inhibition by tryptophan, indicating that the catalytic and regulatory sites are on the same polypeptide chain. Equilibrium dialysis measurements (35) indicate binding of 0.9 mole of tryptophan per mole of component I with a dissociation constant of 40 μM. Inhibition of enzyme activity by tryptophan is competitive with chorismate and noncompetitive with ammonium sulfate. As expected for a single polypeptide chain cooperativity is not observed for binding or inhibition of enzyme activity. It is of interest that anthranilate synthetase Component I is an example of a monomeric regulatory protein. Regulatory enzymes are generally oligomeric (69) and, in fact, anthranilate synthetase in wild type cells is oligomeric (Section III.B).

An important question is whether the tryptophan regulatory site is common or overlapping with the site for chorismate or whether the two sites are distinct. An unequivocal answer cannot yet be given but three lines of evidence suggest that the regulatory site is distinct from the catalytic site. First, in the presence of tryptophan the rate of reaction of anthranilate synthetase

Component I sulfhydryl groups with DTNB was decreased (35), thus suggesting a conformation change upon binding inhibitor. Under appropriate conditions the change in rate of reaction of enzyme sulfhydryl groups with DTNB caused by tryptophan closely paralleled the inhibition of enzyme activity. Tryptophan may provoke a change in conformation that causes inhibition of enzyme activity. Second, cooperativity for inhibition of enzyme activity, binding of tryptophan, and saturation by chorismate in the presence of tryptophan were observed for anthranilate synthetase–PR transferase (18). Such cooperativity is diagnostic for "allosteric" enzymes in which substrate and regulatory effector bind to distinct sites (69). Third, anthranilate synthetase was desensitized to tryptophan inhibition by mutation (13) or when assayed with high concentrations of Mg^{2+} (18). The concentration of tryptophan required for half maximal saturation of the enzyme was increased over seven fold by 5 m*M* $MgCl_2$ (21). Although several interpretations are possible, desensitization of regulatory proteins has been used to argue for distinct catalytic and regulatory sites.

Studies on the kinetics of tryptophan inhibition of enzyme activity (18), inhibition of alkylation by DON (20), and inhibition of glutaminase activity (Section IV) have suggested that tryptophan provokes end-product inhibition by interfering with the binding of either chorismate or glutamine. Direct evidence that tryptophan anatogizes binding of chorismate was recently obtained for the enzyme from *S. typhimurium* (21). Equilibrium dialysis measurements indicated cooperative binding of 1.76 to 1.80 moles of tryptophan per mole of enzyme. Binding was to anthranilate synthetase subunits and not to PR transferase chains as shown by the response to Mg^{2+}. Mg^{2+} antagonizes tryptophan inhibition of anthranilate synthetase (18) but is required for tryptophan inhibition of PR transferase (19). Binding of 1.8 moles of chorismate per mole of anthranilate synthetase–PR transferase with a dissociation constant of 3.6 μM which is equal to the K_m for chorismate was antagonized by tryptophan. In the presence of 7.5 μM tryptophan, chorismate (5 to 48 μM) binding was reduced 90 to 100%. According to various models (69,70) regulatory enzymes may exist in at least two conformational states, each exhibiting preferential affinity for either substrate or

inhibitor. In several cases these expectations have been verified (71–74). A reasonable interpretation of the experiments with anthranilate synthetase–PR transferase from *S. typhimurium* is that cooperative binding of tryptophan to anthranilate synthetase Component I subunits of the oligomer at a distinct regulatory site provokes a conformation change that hinders binding of the first substrate, chorismate. Verification requires *(a)* physical separation of the regulatory and catalytic sites and *(b)* further characterization of putative conformation changes provoked by tryptophan.

VIII. Subunit Interactions and Cooperative Kinetics

Evidence has been given in Section III.B that anthranilate synthetase–PR transferase from *S. typhimurium* is a tetramer of composition I_2II_2. There is thus opportunity for interactions between the identical subunits and between the nonidentical subunits. Both types of interactions have been detected. Interactions between component I chains give positive cooperativity for tryptophan binding (21) and positive cooperativity for Mg^{2+} and chorismate in the presence of tryptophan (18). Apparent negative cooperativity for saturation by $(NH_4)_2SO_4$ ($n = 0.72$) was detected. Although it is recognized that an analysis of kinetics cannot give proof for cooperative interactions (75,76), absence of sigmoidal kinetics with monomeric anthranilate synthetase Components I (35,36) or II (19) clearly indicates the importance of subunit association for sigmoidal kinetics. For this enzyme sigmoidal kinetics is ascribed to subunit interactions. Positive cooperativity for tryptophan inhibition ($n' = 1.6$ to 1.8) deduced from analyses of kinetics has been verified by positive cooperative binding ($n' = 1.4$) (21).

Apparent negative cooperativity for anthranilate in the presence of tryptophan suggests interactions between anthranilate synthetase Component II subunits (19). Curvature in double reciprocal plots suggestive of negative cooperativity for anthranilate was not detected with monomeric Component II or in an aggregate of component II with catalytically inactive anthranilate synthetase Component I (60).

Interactions between the nonidentical subunits is necessitated by ordered binding of substrates (20). Binding of DON or glutamine to anthranilate synthetase component II is preceded by binding of chorismate to component I. The effect of chorismate on tryptophan inhibition of PR transferase activity (19) may illustrate another case for this type of interaction. It is known that chorismate binds to anthranilate synthetase Component I and antagonizes tryptophan inhibition of PR transferase. It is not clear, however, if tryptophan inhibits PR transferase activity by binding to PR transferase or anthranilate synthetase Component I (Section VII). In either case, interactions between the nonidentical subunits are required. In the reciprocal case, tryptophan inhibition of NH_3-dependent anthranilate synthetase activity was facilitated by aggregation of component I with component II (13). Finally, NH_3-dependent anthranilate synthetase activity of component I is increased more than ten fold (35) and the K_m for chorismate decreased five- to sixfold (13) upon aggregation with component II. This is most easily explained by considering that the conformation of component I is favorably changed upon interaction with anthranilate synthetase Component II.

Cooperative kinetics have been detected for anthranilate synthetase–PR transferase from *E. coli* (14) and for anthranilate synthetase from *S. marcescens* (9), *B. alvei* (25), and *C. violaceum* (6).

IX. Possible Physiological Function of Multienzyme Complexes

Aggregates of anthranilate synthetase with PR transferase (type II) or with other enzymes of tryptophan synthesis (type III) may function to catalyze multiple reactions without release of free intermediates. If the aggregates so far detected are fragments of larger but more fragile multienzyme complexes, overall conversion of chorismate to tryptophan may occur without release of free intermediates. The extreme lability of *N*-(5′-phosphoribosyl)anthranilate at pH 7.1 and 37° (77) suggests that this free intermediate may not normally exist. In *E. coli, A. aerogenes,* and *S. typhimurium* the initial reactions of the branched pathways to phenylalanine, tyrosine, and tryptophan are all

catalyzed by bifunctional enzymes or multienzyme complexes (78,79) suggesting three "channeled" pathways diverging from chorismate. Each multifunctional enzyme or enzyme aggregate is subject to feedback inhibition by the amino acid end product.

Some recent evidence supports the speculation of channeling in the tryptophan pathway of *N. crassa*. Using a multienzyme aggregate containing anthranilate synthetase, *N*-(5′-phosphoribosyl) anthranilate isomerase, and indole 3-glycerol phosphate synthetase activities, Gaertner et al. (80) showed that the maximal rate of indole 3-glycerol phosphate synthesis from *N*-(5′-phosphoribosyl) anthranilate was approximately twofold greater than that obtained using 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate (CDRP). Little or no free 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate was detected in the conversion of *N*-(5′-phosphoribosyl)anthranilate to indole 3-glycerol phosphate. This increase in catalytic efficiency was called "catalytic facilitation" and its possible relationship to metabolic pool separation or channeling *in vivo* was noted.

In extracts of *S. typhimurium* up to 60% of the anthranilate synthetase–PR transferase may be particulate (26). A curious property of the particulate enzyme was that its PR transferase activity was latent although component II, as measured by glutamine-dependent anthranilate synthetase, was fully active. PR transferase activity was "unmasked" upon solubilization of anthranilate synthetase–PR transferase. It remains to be determined if this is another example of "catalytic facilitation."

X. Relationship to Other Glutamine Amidotransferases

Anthranilate synthetase enzymes of type I and II are similar to other glutamine amidotransferases (3,44–52) in the following respects: *(a)* utilization of either glutamine or NH_3, *(b)* selective inactivation of glutamine reactivity by treatment with glutamine analogs or sulfhydryl reagents, *(c)* presence of glutaminase activity, and *(d)* oligomeric subunit composition. Formylglycinamide ribonucleotide amidotransferase appears to be the only glutamine amidotransferase for which evidence supporting a single polypeptide chain has been reported (81).

Selective inactivation of glutamine-dependent activity implies separate sites for glutamine and NH_3. Recent work by Levitzki and Koshland (82) indicates that "nascent" NH_3 is formed upon glutamylation of cytidine triphosphate synthetase. This NH_3 is used for amination of uridine triphosphate without dissociating from the enzyme. Furthermore, added NH_3 and NH_4^+ from solution interact with the same enzyme site as "nascent" NH_3 generated from glutamine. Recent experiments with chicken liver formylglycinamide ribonucleotide amidotransferase (83) also suggest utilization of the amide of glutamine via the NH_3 site.

An important question is whether or not the glutamine binding site is on the same polypeptide chain as the site for NH_3. Stated another way this question is whether other glutamine amidotransferases, besides anthranilate synthetase, contain nonidentical subunits. Levitzki, Stallcup, and Koshland (54) have provided strong evidence that cytidine triphosphate synthetase is an oligomer of identical subunits. Formylglycinamide ribonucleotide amidotransferase from chicken liver appears to be a single polypeptide chain (81) and therefore could not contain a subunit specific for binding glutamine. Phosphoribosylpyrophosphate amidotransferase from pigeon liver (84) contains subunits of apparently identical size, and therefore a specific subunit for binding glutamine may be unlikely.

On the other hand, in addition to anthranilate synthetase, carbamyl phosphate synthetase (85), and the enzyme system required for synthesis of 4-aminobenzoate (86) contain nonidentical subunits. *E. coli* carbamyl phosphate synthetase has recently been characterized (85) as an oligomer containing subunits of molecular weight approximately 130,000 and 42,000. In the absence of positive effectors ($s^\circ_{20,w} = 7.35$) the enzyme contained one subunit of each type. Positive effectors promoted association to an oligomer of about 16 S. Separation of active subunits was achieved by gel filtration in 1.0 *M* potassium thiocyanate. The isolated heavy subunit retained the capacity for NH_3-dependent carbamyl phosphate synthesis and two partial reactions, but was inactive with glutamine. Sites for positive and negative effectors were localized to the large subunit. The small subunit retained glutaminase activity and restored glutamine reactivity when recombined with the large protein chain. It was proposed that

glutamine binds to the small subunit and the amide is transferred to the catalytic site on the large subunit. The large and small subunits are therefore formally analogous to anthranilate synthetase Components I and II, respectively.

Several glutamine amidotransferases (82,83,85) in addition to anthranilate synthetase (9,20) have glutaminase activity under conditions that the overall reaction cannot occur. It has been suggested that this activity is a reflection of amide transfer from the glutamine to ammonia sites. (9,20).

Since anthranilate synthetase Component II functions as a glutamine binding protein, the question arises if this subunit could function with other glutamine amidotransferases. Kane et al. (11) have approached this question with the anthranilate synthetase system in *B. subtilis*. Growth of a mutant strain of *B. subtilis* (*trp*X$^-$) with defective anthranilate synthetase Component II was inhibited by low concentrations of tryptophan. Inhibition was reversed by folate or 4-aminobenzoate, suggesting that repression by tryptophan of residual anthranilate synthetase Component II synthesis starved cells for the vitamin. Growth of the *trp*X$^-$ strain exhibited increased sensitivity to inhibition by the 4-aminobenzoate analogue sulfathiazole. Finally the glutamine-dependent enzyme required for 4-aminobenzoate synthesis was reduced from a specific activity of 4.0 in an extract of a strain wild type for *trp*X to <0.1 in an extract of the *trp*X$^-$ strain. On the other hand, there was no difference in CTP synthetase activity between the two strains and the *trp*X$^-$ strain was not hypersensitive to the histidine antimetabolite 1,2,4-triazole-3-alanine. These results suggest that in *B. subtilis* anthranilate synthetase Component II functions in the synthesis of 4-aminobenzoate in addition to anthranilate but not for the synthesis of CTP or histidine.

E. coli contains two glutaminases designated A and B. Glutaminase A appears in stationary phase cells and has a pH optimum of 5 (87), while glutaminase B is in log phase cells and has a broad pH optimum above 7 (88). The possible relationship of glutaminase B, molecular weight about 80,000 (89), to the glutaminase activity of glutamine amidotransferases including anthranilate synthetase has been considered by Prusiner and Stadtman (88). Preliminary results have not detected a relationship

between glutaminase B and several of the amidotransferases (Prusiner, personal communication). Likewise, it appears that the glutamine binding protein of *E. coli* implicated in glutamine transport may not be related to glutamine amidotransferases, since the former is devoid of cysteine and is therefore unaffected by DON (90).

XI. Speculations on Evolutionary Relationships

Glutamine amidotransferases may have evolved from simple monomeric NH_3-dependent enzymes. Following the evolution of glutamine, primitive NH_3-dependent enzymes were perhaps modified by aggregation to a second subunit with the specialized function of glutamine binding and amide transfer to the existing site for NH_3 (9,20,59,83,85). In some cases fusion of contiguous genes may have led to formation of bifunctional glutamine amidotransferases in which the sites for NH_3 and glutamine were covalently joined in a single protein chain as for CTP synthetase (54) and formylglycinamide ribonucleotide amidotransferase (81). With other enzyme reactions the gene for the putative glutamine binding protein may have either remained distinct, as for type I anthranilate synthetase or *E. coli* carbamyl phosphate synthetase, or it may have been inserted into a chromosomal site contiguous with a preexisting gene for an enzyme of the pathway such as PR transferase. Gene fusion in the latter case could then produce a bifunctional PR transferase as in *E. coli* (37) and *S. typhimurium* (26,59).

The experiments on trypsin digestion (26,59) of anthranilate synthetase–PR transferase from *S. typhimurium* can be viewed in this highly speculative context. A schematic representation for the effect of trypsin on anthranilate synthetase–PR transferase from *Salmonella* is shown in Figure 5. The native enzyme is shown as a tetramer containing two chains of anthranilate synthetase Component I and two chains of component II. In this hypothetical scheme, anthranilate synthetase Component II is bifunctional. An NH_2-terminal segment of approximately 25% concerned with glutamine binding is covalently joined to a region containing the catalytic site for PR transferase. The PR transferase portion and the connecting region are highly sensitive to