

Molecular Studies on Enzymes in Chorismate Metabolism and the Enterobactin Biosynthetic Pathway

CHRISTOPHER T. WALSH,* JUN LIU, FRANK RUSNAK, and MASAHIRO SAKAITANI

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Received February 26, 1990 (Revised Manuscript Received June 15, 1990)

Contents

I. Introduction	1105
II. Enzymes Utilizing Chorismate as Substrate	1105
A. Chorismate Mutase	1107
B. Anthranilate Synthase	1108
C. PABA Synthase	1109
D. Isochorismate Synthase	1110
1. Michael Addition/Elimination Sequence	1112
2. Covalent Catalysis with a Nucleophile from the Enzyme Active Site	1112
3. Covalent Catalysis with a Nucleophile from the Substrate Itself: The Carboxylate in the Enol Pyruvyl Side Chain	1113
4. Concerted Mechanism Involving a Magnesium-Bound Transition State	1113
E. Chorismate Lyase	1113
III. Mechanistic Studies in the Biosynthesis of Enterobactin	1114
A. Introduction to Siderophores: Enterobactin	1114
1. Chemical Properties of Enterobactin	1114
2. Synthetic Strategies for the Macrocycle Enterobactin	1115
3. Metabolism of Enterobactin in <i>Escherichia coli</i>	1115
B. Genetic Organization and Regulation of Enterobactin Metabolism	1117
C. Biosynthesis of the 2,3-Dihydroxybenzoate Moiety of Enterobactin	1118
1. Molecular Biological Strategy	1118
2. Isochorismate Synthase	1119
3. Chemical Properties of Isochorismic Acid	1119
4. Isochorismatase	1120
5. 2,3-Dihydro-2,3-dihydroxybenzoate Dehydrogenase	1122
D. Biosynthesis of Enterobactin from L-Serine and 2,3-Dihydroxybenzoate	1124
IV. Other Transformations	1127

I. Introduction

The shikimic acid pathway is used by microorganisms to biosynthesize the aromatic amino acids phenylalanine, tryptophan, and tyrosine, folate coenzymes, vitamins such as the benzenoid and naphthenoid coenzymes Q and vitamin K, and a broad range of aromatic secondary metabolites, including the 2,3-dihydroxybenzoate-containing chelating molecules for iron uptake and accumulation. The common branch point of the pathway is at the dihydroaromatic compound chorismate 1. As shown in Figure 1, chorismate is converted by five separate enzymes to prephenate (2),

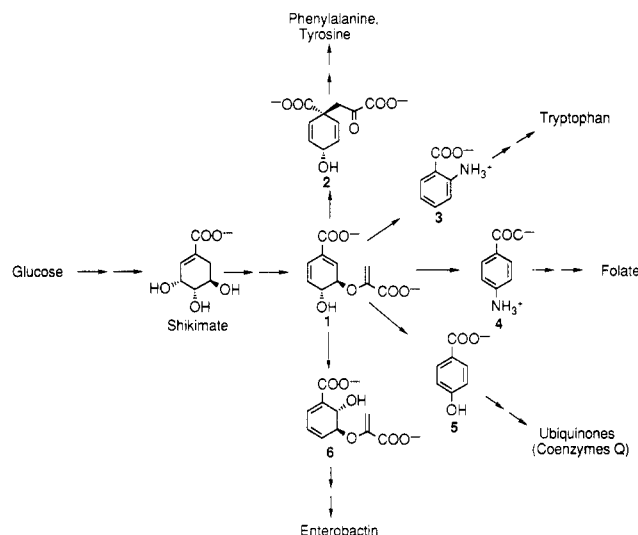


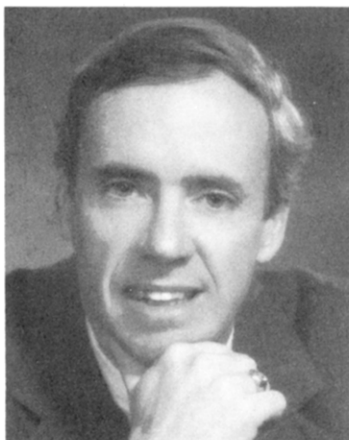
Figure 1. Metabolic roles of chorismic acid.

anthranilate (3), *p*-aminobenzoate (4), *p*-hydroxybenzoate (5), and isochorismate (6). This set of three aromatic and two dihydroaromatic metabolites comprises the first committed precursors in the biogenesis of such products as phenylalanine and tyrosine, tryptophan, chloramphenicol, coenzyme Q, and catechol-containing molecules such as enterobactin.

While the general outlines of these pathways and the structures of intermediates have been worked out by more or less classical biosynthetic studies,¹⁻³ other than in the phenylalanine and tyrosine pathway, rather little has been known about the mechanistic features of the five initial transformations of chorismate depicted in Figure 1 or about the structure function characteristics of the enzymic catalysts. In the past few years substantial progress has been made in molecular characterization of some of these enzymes and their mechanisms, and these advances will be summarized in this paper. The advent of molecular biology, including such techniques as gene cloning, sequencing, expression, and protein overproduction, has begun to revolutionize the knowledge base in enzymology, and the multistep pathway for conversion of chorismate via isochorismate and 2,3-dihydroxybenzoate to the cyclic (dihydroxybenzoyl)serine trilactone enterobactin will be analyzed to exemplify these powerful approaches in the second part of this paper. The reader is referred to authoritative reviews on other aspects of the shikimate pathway.^{2,4,5}

II. Enzymes Utilizing Chorismate as Substrate

The key branch point metabolite chorismate is generated from the precursor enolpyruvylshikimate phos-



Christopher Walsh received his B.A. in biology from Harvard University in 1965. He went on to receive his Ph.D. in life sciences from The Rockefeller University in 1970 and then pursued postdoctoral studies as a Helen Hay Whitney Foundation Fellow in the Graduate Department of Biochemistry at Brandeis University. In 1972 he accepted a position as Assistant Professor of chemistry and biology at MIT, remaining there to become the chairman of the Chemistry Department from 1982 to 1987. In 1987 he accepted the position of Chairman of the Department of Chemistry and Molecular Pharmacology at Harvard Medical School where he is currently David Wesley Gaiser Professor. Professor Walsh's research interests include the study of enzymatic reaction mechanisms, design of suicide substrates and other mechanism-based enzyme inactivators, flavoenzymes and other redox enzymes, enzyme stereochemistry, and molecular toxicology. Professor Walsh has been a fellow of the Alfred P. Sloan Foundation and a Dreyfus Teacher-Scholar. A member of the National Academy of Sciences and the American Academy of Arts and Sciences, he is the author of over 240 scientific publications.



Jun Liu obtained his B.S. degree in 1983 from Nanjing University. He went to The Ohio State University in 1984 and received an M.S. degree in organic chemistry in 1986, working under Professor David Hart. He moved to Boston that same year and did his graduate work under the joint supervision of Professor Christopher Walsh and Professor Glenn Berchtold, receiving his Ph.D. degree in chemistry from the Massachusetts Institute of Technology in 1990. After a short summer postdoctoral stint with Professor Walsh at Harvard Medical School, he has been a postdoctoral fellow in Professor Stuart Schreiber's group at the Chemistry Department of Harvard University. His research interests lie between chemistry and biology, including biological catalysis, immunoorganic chemistry, and design of enzyme-like catalysts for organic synthesis.

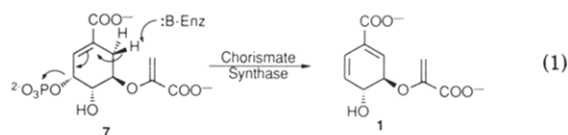
phate (EPSP, **7**) by a net 1,4-elimination of inorganic phosphate by the enzyme chorismate synthase, setting up the diene system. The chorismate synthases from *Neurospora crassa*⁶ and *Bacillus subtilis*⁷ have been purified to apparent homogeneity while the *aroC* gene encoding for *Escherichia coli* chorismate synthase has recently been cloned, sequenced, overproduced, and



Frank Rusnak obtained his B.S. degree in biochemistry from the University of Minnesota in 1982. After 1 year studying bacterial degradation of aromatic compounds with Professor Peter Chapman and Professor Stanley Dagley at the University of Minnesota, he moved to the Gray Freshwater Biological Institute as a NIH predoctoral trainee, receiving his Ph.D. with Professor Eckard Münck investigating the spectroscopic properties of metalloproteins. He then joined Professor Christopher Walsh's group as an NIH postdoctoral fellow where he worked on the enzymes of enterobactin biosynthesis. Dr. Rusnak will be joining the Hematology Department at the Mayo Clinic and Foundation. His interests include spectroscopic and molecular aspects of metalloproteins and enzymes in metal utilization.



Masahiro Sakaitani received a B.S. in chemistry from Kwansei-gakuin University in 1983, an M.S. in chemistry from Osaka University (Sunbor Fellow) in 1985, and a Ph.D. in chemistry from Osaka University in 1989. After receiving the M.S., he joined the Suntory Institute for Bioorganic Research (directed by K. Nakanishi). He is currently on a 2-year sabbatical visit to Harvard Medical School with Professor Christopher Walsh.



purified.⁸ The enzymic reaction occurs via an unconventional trans-1,4-elimination.⁹⁻¹¹ Several mechanistic proposals have been examined.¹⁰⁻¹³ The mechanism of the enzymic reaction, especially the roles of the reduced flavin and NADPH, however, remained poorly understood. The unusual enolpyruvyl ether group in chorismate and EPSP was put in place by the preceding enzyme EPSP synthase, utilizing phosphoenolpyruvate as the donor to the 5-alcohol moiety of shikimate. The highly functionalized dihydroaromatic diacid chorismate is poised to undergo directed flux to rearranged

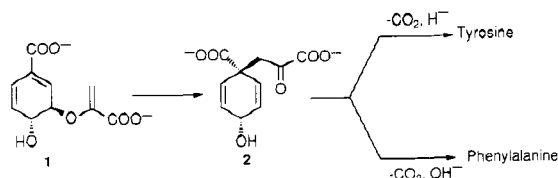


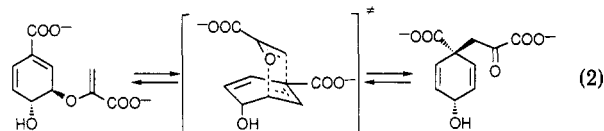
Figure 2. Metabolic routes of chorismate to tyrosine and phenylalanine via prephenate.

dihydroaromatic products, e.g., the famous net 3,3-rearrangement to prephenate and the net 1,5-isomerization to isochorismate, or to regiospecific ortho- or para-substituted aromatic products. Each of the five enzymes carrying out these transformations displays exquisite specificity as the gate keeper to separate metabolic pathways. Chorismate mutase appears to carry out a unique transformation while the other four enzymes, anthranilate synthase, PABA synthase, chorismate lyase, and isochorismate synthase, share some overlapping mechanistic and perhaps structural features.

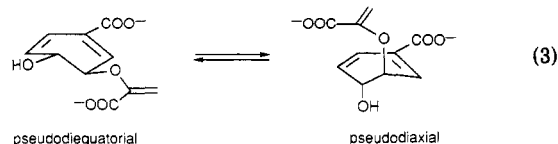
A. Chorismate Mutase

The most celebrated and best characterized of the chorismate-processing enzymes has been chorismate mutase, attracting justly deserved attention, not only for its centrality in producing the precursor to the essential aromatic amino acids phenylalanine and tyrosine but also as the sole catalyst in primary metabolism engaged in an apparent pericyclic reaction, the Claisen rearrangement to prephenate (2), (Figure 2). In part because this enzyme has been extensively studied mechanistically, it is not a primary focus of this chapter and the large body of chemical and enzymatic evidence that has accrued to rule out or support the many mechanistic hypotheses that have been advanced^{14,15} will not be reviewed in detail.

Knowles and his colleagues have established several constraints on the enzymic mechanism by an elegant series of stereochemical, spectroscopic, and isotopic kinetic and product effect analyses. They have established that the mutase catalysis proceeds via a chairlike transition state rather than a boatlike transition state.¹⁶



Further, they have established by ¹H NMR analysis that, in aqueous solution at 25 °C, while the pseudodiequatorial conformer of chorismate predominates, as expected, some 10–40% of the molecules exist in the pseudodixial conformer that is the species set up for the 3,3-rearrangement.¹⁷ Thus, the mutase will be able



to select this minor conformer directly from solution without the need for induced conformational isomerization in the active site. The net rate acceleration of the enzyme over the uncatalyzed Claisen rearrangement

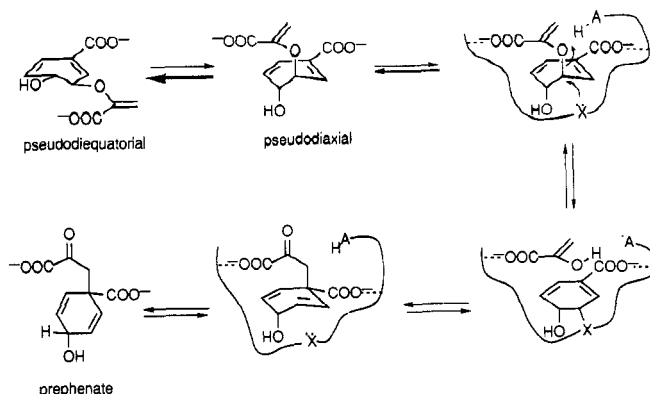
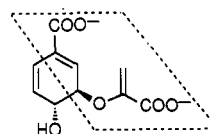


Figure 3. One possible mechanism of chorismate mutase catalyzed 3,3-rearrangement of chorismate.

CHART I



in comparable circumstances is about 10⁶-fold,¹⁸ and the enzyme's catalytic efficiency, k_{cat}/K_m , has been reported as ca. $1.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.¹⁹ From a series of primary and secondary deuterium and tritium isotope effect studies with regio- and stereospecifically labeled chorismates. Knowles and colleagues have reasoned that the rate-limiting step in chorismate mutase catalysis is the heterolytic cleavage of the oxygen–carbon bond in the ether linkage with the generation of a transient species with substantial dipolar character rather than a synchronous, concerted 3,3-rearrangement.^{14,20} One mechanistic proposal involves general-acid-catalyzed protonation of the vinyl ether oxygen with attack by an enzyme nucleophile at C₅ to yield a transient intermediate with the cyclohexadienecarboxylate moiety covalently tethered to the enzyme. Reattack by the enolpyruvate fragment at the C₁-position of the cyclohexene would lead by an S_N2' route to the nascent prephenate product as shown in Figure 3, redrawn from Guilford et al.¹⁴ So far, however, the existence of the putative nucleophile has not been established. Pawlak et al.²¹ have further delineated the substrate recognition requirements for chorismate mutase, showing that neither the 5,6-olefinic nor the 4-OH group is absolutely necessary, reducing the minimal features for rearrangement to the allyl vinyl ether functionality and both carboxylates as shown (Chart I).

Most versions of bacterial chorismate mutase have been detected as part of a bifunctional polypeptide chain with fusion to either prephenate dehydrogenase or prephenate dehydratase,²² catalyzing the decarboxylative aromatization and loss of either the C₄-hydrogen (such as a hydride ion transfer to NAD⁺) or the C₄-hydroxyl to yield L-tyrosine or L-phenylalanine, respectively (Figure 4). Structure/function studies of the mutase portion of these mutase/dehydrogenase or mutase/dehydratase enzymes have been hampered by this bifunctionality, but a small (14.5-kDa) monofunctional chorismate mutase has recently been purified to homogeneity²³ and is the object of choice for further structural and functional characterization.

Based on the chairlike transition state, a series of transition-state analogue inhibitors has been syntheses-

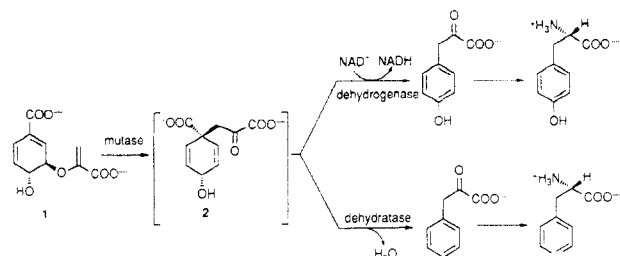
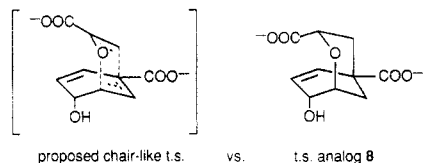


Figure 4. Chorismate mutase as part of bifunctional enzymes.

CHART II

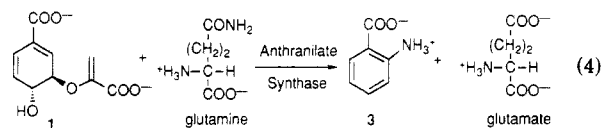


ized and evaluated.²⁴⁻²⁶ Among the inhibitors of chorismate mutase, the most potent one is the endo isomer of oxa bicyclic diacid 8 (Chart II) made by Bartlett and colleagues with $K_i/K_m = 0.008$.²⁶ The fact that this transition-state analogue inhibits chorismate mutase lends support to the proposed chairlike transition state.

Using the transition-state analogue 8 as a hapten, two groups^{27,28} have recently reported successful generation of catalytic antibodies able to accelerate the chorismate to prephenate conversion. The antibodies show saturation kinetics, and one yields $k_{cat}/k_{uncat} = 250$ ²⁸ while the second gives $k_{cat}/k_{uncat} = 10^4$,²⁷ with a k_{cat} of 2.7 min⁻¹ and a K_m of 260 μ M at 10 °C, pH 7.0. The rate acceleration by the latter monoclonal antibody is only ca. 100-fold less than that of chorismate mutase itself! The catalytic efficiency of this antibody ($k_{cat}/K_m = 1.7 \times 10^2$ M⁻¹ s⁻¹), however, is far less than that of the naturally occurring enzyme ($k_{cat}/K_m = 1.0 \times 10^6$ M⁻¹ s⁻¹).

B. Anthranilate Synthase

Anthranilate synthase, the first step specific to tryptophan biosynthesis, catalyzes a net regiospecific amination/aromatization sequence on chorismate. In



most bacteria the enzyme is composed of two non-identical subunits referred to as component I (AS-I) and component II (AS-II) encoded by the *trpE* and *trpG* genes, respectively. The TrpE subunit catalyzes the NH₃-dependent conversion of chorismate to anthranilate,²⁹ while the TrpG subunit has glutaminase activity, molecular weight 20 000, that hydrolyzes cosubstrate glutamine, via a γ -glutamyl-S-cysteinyl enzyme intermediate³⁰ to release nascent NH₃ for the amination sequence. The genetic organization of Trp operons from different organisms show great diversity. In some bacteria such as *Serratia marcescens*,³¹ *Pseudomonas putida*,³² *Pseudomonas aeruginosa*,³³ and *Thermus thermophilus*³⁴ a single *trpG* gene encodes the AS-II subunit. In others such as *E. coli*, *Salmonella typhimurium*, and *Shigella dysenteriae*^{35,36} *trpG* is fused to

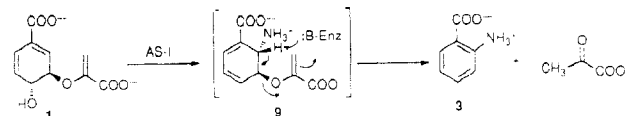


Figure 5. Amino diene intermediate in anthranilate synthase catalyzed conversion of chorismate to anthranilate.

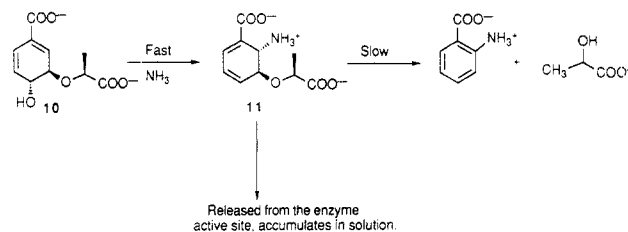


Figure 6. Accumulation of the amino diene intermediate from a chorismate analogue with an (*R*)-lactyl side chain.

the *trpD* gene, encoding for anthranilate phosphoribosyltransferase, the second enzyme in the tryptophan pathway, while in *Rhizobium meliloti* a single gene encodes both AS-I and AS-II activities, apparently evolving from fusion of *trpE* and *trpG* genes.³⁷ Historically the enzyme from *S. marcescens* was studied most widely because a separate TrpG polypeptide is generated and anthranilate synthase composed of AS-I and AS-II can be purified in quantity to homogeneity and studied mechanistically.^{38,39}

The most likely intermediate, working backward from the aromatic *o*-aminobenzoate product, had been the amino diene 9, *trans*-6-amino-5-[(1-carboxyethenyl)-oxy]-1,3-cyclohexadiene-1-carboxylate, an "amino" analogue of isochorismate (Figure 5). Berchtold and colleagues synthesized this proposed intermediate as the *trans* aminoenolpyruvyl species, and we demonstrated its ammonia independent chemical and kinetic competence to undergo conversion⁴⁰ to anthranilate with pure enzyme, consistent with a *cis* elimination/aromatization route and validating enzyme recognition of the intermediate when added from solution. Independent experiments done by Teng and Ganem⁴¹ confirmed these observations. Attempts to demonstrate accumulation of the amino diene in catalytic turnover of chorismate did not succeed, perhaps reflecting that aromatization may be non-rate-limiting.

To probe further for accumulation of aminocyclohexadiene intermediates, we turned to synthesis of alternate substrates for the enzyme and determined that modifications of the C₃ enolpyruvyl ether substituent to a C₃ (*R*)- or (*S*)-lactyl ether or a C₃ glycolyl ether led to 10–20-fold decreases in k_{cat} values compared to chorismate.⁴² Anthranilate synthase displayed a 12/1 of *S/R* lactyl ether preference by k_{cat}/K_m criteria such that the (*S*)-lactyl analogue 10 was processed at 1/81 the catalytic efficiency of chorismate while the *R* side chain analogue was at 1/872 (Figure 6). UV-visible monitoring of the incubation solutions on these lactyl analogues indicated transient accumulation of the aminocyclohexadiene species before further conversion to anthranilate. This was validated with the (*R*)-lactyl analogue of chorismate 10 by quenching the enzymic incubation, HPLC isolation of a new species 11, and its NMR and UV characterization. On reincubation with the enzyme, but now in the absence of NH₃, chorismate formation was detected. By both k_{cat}/K_m criteria and the isolation of the intermediate amino diene, it is likely

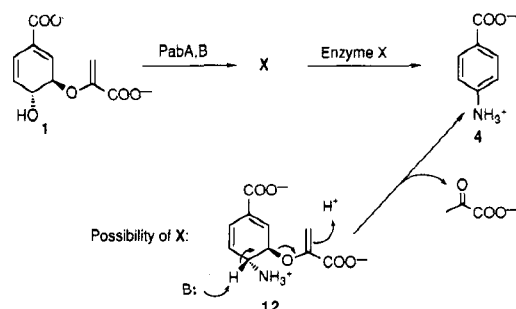


Figure 7. Possible role of putative enzyme X as a 4-amino-4-deoxychorismate lyase.

that aromatization has been selectively slowed by the use of a poorer leaving group in the elimination step (lactyl vs enolpyruvyl ether).

At this juncture the major mechanistic issue with anthranilate synthase is the mode of formation of the amino analogue of isochorismate. Both stepwise and concerted mechanisms are possible; as there is yet no evidence distinguishing between these alternatives, the issue is deferred until the discussion below on isochorismate synthase where a homologous enzyme faces a highly analogous mechanistic challenge and the same solution may likely apply for both anthranilate synthase and isochorismate synthase.

C. PABA Synthase

It has long been assumed that for the biogenesis of *p*-aminobenzoate, the precursor to the folate coenzymes, paralleled the biosynthesis of its regioisomer *o*-aminobenzoate (anthranilate), bolstered by the genetic evidence for two unlinked genes *pabA* and *pabB* needed for the regiospecific amination/elimination/aromatization sequence presumed for chorismate and cosubstrate glutamine. Indeed, *pabA* encodes a 21.7-kD polypeptide and *pabB* a 53.4-kD polypeptide in *E. coli*, and gene sequencing demonstrated homology of the *pabA* sequence with *trpG*, encoding glutaminase subunit of anthranilate synthase⁴³ while corresponding homology was found between *trpE* and *pabB* encoded chorismate aminating subunits.⁴⁴

However, the protein biochemistry and mechanistic enzymology studies on PABAS have been much less advanced due to an inability to detect and then purify active PABA synthase. At first this was attributed to low levels of wild-type activity, but overproduction of the PabB subunit to 30% purity yielded only a very low specific activity for PABAS as assayed by specific fluorescence of the aromatic product.⁴² Some of the mystery may have been solved in recent studies by Nichols et al. in which they separately overproduced component I some 273-fold and component II 566-fold from a two-plasmid construction expressed in the same cell, yet again found almost no activity until they added an ammonium sulfate extract from an *E. coli* cell, BN117, that lacks both PabA and PabB.⁴⁵ The extract contains a protein required in addition to components I and II to make PABA and has been designated enzyme X. Nichols et al. have used this three-protein system to show that components I and II use glutamine and chorismate to produce a diffusible intermediate that is then converted by enzyme X to PABA (Figure 7). No information was provided on the nature of the intermediate or the specificity of enzyme X, but puri-

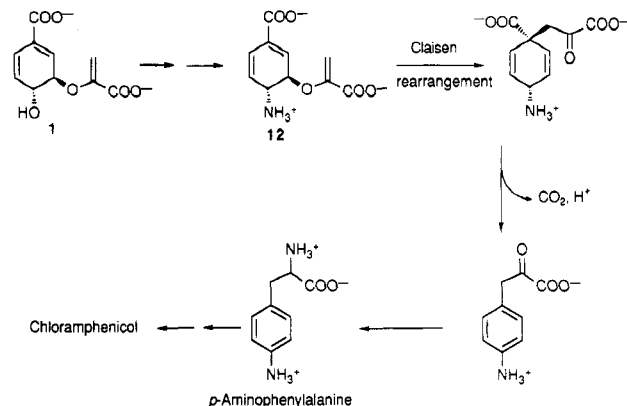


Figure 8. 4-Amino-4-deoxychorismate as a precursor to chloramphenicol.

fication of the putative intermediate should occur rapidly. The speculation was that 4-amino-4-deoxychorismate (12) is the intermediate and enzyme X may be an aromatizing activity as noted below. A key conclusion is that PabA and PabB do not in fact make PABA but probably a dihydroaromatic amino analogue of chorismate and, in that sense, act as a hybrid catalyst between the two-subunit anthranilate synthase, discussed above, and the one-subunit isochorismate synthase discussed below.

The implication of 4-deoxy-4-aminochorismate (12) as an intermediate in PABA biosynthesis arises from its synthesis by Teng et al.⁴⁶ and their demonstration of its processing in crude extracts to PABA. One now anticipates this amino diene is the product of PabA and PabB action on chorismate and is in turn a substrate for elimination and aromatization by enzyme X. A major remaining chemical question is how chorismate is converted to 4-amino-4-deoxychorismate, a net 1,1-replacement of NH₂ for OH. Teng et al.⁴⁶ had tested free isochorismate as a precursor of PABA in crude extract, and the lack of detectable product ruled against an enzymic sequence of chorismate to isochorismate to 4-amino-4-deoxychorismate in a series of 1,5-addition/eliminations involving free intermediates. The identical chirality at the C₄ in 4-amino-4-deoxychorismate intermediate and chorismate itself ruled out a direct 1,1-displacement of OH by NH₂. To overcome the stereochemical dilemma, an alternative may involve covalent addition by an enzyme nucleophile in an S_N2 (1,1) or S_N2' (1,3) or a double-S_N2' (1,5) mechanism which would accomplish the expulsion of the 4-OH substituent, tether a diene intermediate in the active site, and then be subject to readdition not of OH₂ but of NH₃ to yield the 4-amino-4-deoxychorismate with the anticipated stereochemistry (for a more detailed discussion of this possibility, see section II.D). No evidence yet exists for such an intermediate, but the pure PabB polypeptide has not yet been examined mechanistically. We will return to consideration of this type of sequence in the isochorismate synthase discussion. Attempts to inhibit PabB with electrophilic substrate analogues did not cause time-dependent inactivation.⁴²

Teng et al.⁴⁶ also used synthetic 4-amino-4-deoxychorismate (12) in an experiment with partially purified arylamine synthetase, an enzyme known in *Streptomyces* to produce *p*-aminophenylalanine for the biogenesis of the antibiotic chloramphenicol^{47,48} (Figure 8). While little enzymology has been conducted on this

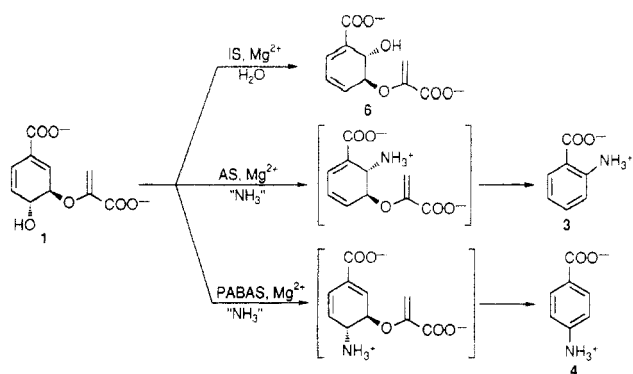


Figure 9. Comparison of isochorismate synthase with anthranilate synthase and PABA synthase.

unstable three-protein complex,^{47,48} the utilization of 4-amino-4-deoxychorismate supports the following mechanistic proposal in which this molecule is an early intermediate that then undergoes a 3,3-rearrangement (an amino version of chorismate mutase (vide supra)) and a decarboxylative hydride transfer and aromatization (an amino version of prephenate dehydrogenase) sequence.

D. Isochorismate Synthase

A third enzyme with clear structural and mechanistic homology to anthranilate synthase and PABA synthase is isochorismate synthase, encoded by the *entC* gene, initiating the enterobactin biosynthetic pathway in enteric bacteria, as discussed in the second half of this review. Similar to anthranilate synthase (and indirectly to PABA synthase), isochorismate synthase catalyzes the 1,5 double- S_N2' displacement of the 4-hydroxyl group in chorismate with water to give the dihydroaromatic diene isochorismate. Unlike its two counterparts, however, isochorismate synthase does not catalyze the further aromatization of the resultant isochorismate, making it an attractive target for the elucidation of the mechanism of catalysis (Figure 9).

Isochorismate synthase (EntC) and its role in the biosynthesis of 2,3-dihydroxybenzoate leading to the siderophore enterobactin (enterochelin) were first discovered by Gibson and co-workers.^{49,50} The EntC activity converting chorismate to isochorismate had been detected in crude extracts by Gibson et al.,⁵¹ but it had not been substantially purified. Further while the *entC* gene had been mapped within a cluster of other *ent* biosynthetic genes,^{52–55} in fact the fine structural mapping was incorrect. When we subcloned the putative “*entC*” gene, expressed and purified its encoded protein to homogeneity, it had no detectable isochorismate synthase activity but instead was 2,3-dihydroxybenzoate dehydrogenase, the *entA* gene product that catalyzes the third step in the pathway.⁵⁶ Subsequent sequencing of the nearby “*fepF*” gene by Elkins and Earhart⁵⁷ and Ozenberger et al.⁵⁸ revealed an ORF with 19% identity to *pabB* and 22% identity to *trpE*. This turned out to be the real *entC* gene, leading to the revised gene order of Figure 23 (section III.B) and permitting us to subclone and generate a useful overproducer by a site-specific procedure that leads to high expression of the cloned gene.⁵⁹ Thus, we could purify isochorismate synthase to homogeneity and begin its initial characterization. In turn, the availability of pure EntC in quantity has permitted the production and rapid HPLC

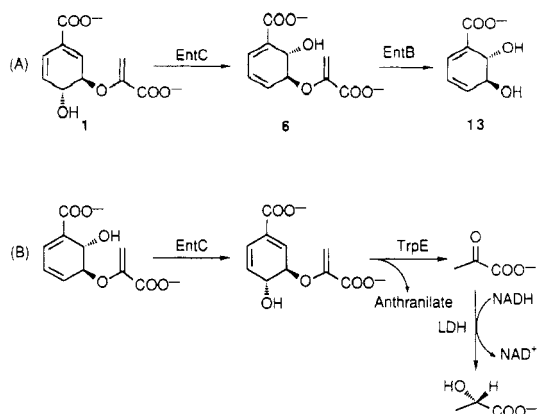
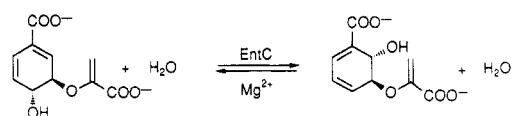


Figure 10. Continuous spectrophotometric assay of isochorismate synthase.



$$K_{eq} = \frac{[\text{Isochorismate}]}{[\text{Chorismate}]} = \frac{V_{maxf}}{V_{maxr}} \cdot \frac{K_{mr}}{K_{mf}}$$

Figure 11. Equilibrium constant between chorismate and isochorismate calculated from Haldane's equation.

purification of isochorismate, an otherwise difficult molecule to obtain.

Isochorismate synthase is active as a monomer and is capable of catalyzing the ready interconversion of chorismate and isochorismate. To study the mechanism of isochorismate synthase, a reliable and sensitive method of assay was crucial. Of the two methods available in the literature for this enzyme assay,⁵¹ one involved an estimation of the remaining chorismate in an incubation mixture by conversion of chorismate to anthranilate while the other was via isochorismate decomposition to salicylate on heating assuming that ca. 25% of the isochorismate gave rise to salicylate.⁵¹ These methods are tedious, and they do not allow quantitative assessment of kinetic constants such as k_{cat} and K_m . Taking advantage of the simultaneous purification of isochorismatase (EntB), an enzyme immediately following EntC in the *ent* pathway and catalyzing the removal of the enolpyruvyl side chain of isochorismate to give 2,3-dihydro-2,3-dihydroxybenzoate (13) (detailed in the second half of this review), and the known extinction coefficients of both chorismate ($\lambda_{max} = 273$ nm, $\epsilon = 2630$ cm⁻¹ M⁻¹)⁶⁰ and 2,3-dihydro-2,3-dihydroxybenzoate ($\lambda_{max} = 278$ nm, $\epsilon = 8150$ cm⁻¹ M⁻¹),⁶¹ a continuous spectrophotometric assay was developed as shown in Figure 10A. Since isochorismate synthase catalyzes the reversible interconversion of chorismate and isochorismate, an assay for the reverse direction is also needed. This is accomplished in a similar fashion as the forward direction by coupling the reverse reaction with anthranilate synthase (Figure 10B) and lactate dehydrogenase. With these two assays, the kinetic constants were determined with $K_m = 14$ μ M and $k_{cat} = 173$ min⁻¹ for chorismate in the forward direction and $K_m = 5$ μ M and $k_{cat} = 108$ min⁻¹ for isochorismate in the reverse direction.

Given that the isochorismate synthase catalyzed reaction is in a formal sense a one-substrate/one-product reaction, the thermodynamic constants can be derived

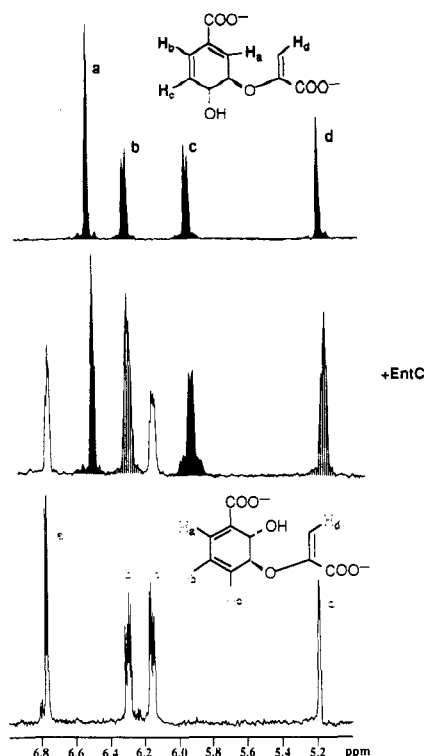


Figure 12. ¹H NMR spectra of chorismate (top), isochorismate (bottom), and an equilibrium mixture of both in the presence of isochorismate synthase (middle).

from the kinetic constants by the Haldane equation⁶² as shown in Figure 11. A K_{eq} of 0.56, favoring chorismate, was obtained. This corresponds to a free energy difference of 0.36 kcal/mol between chorismate and isochorismate with isochorismate slightly higher in energy. Considering that isochorismate has a cyclohexadiene fully conjugated with the carboxylate while chorismate has a cross-conjugated system, one might have expected that isochorismate should be slightly more stable as opposed to what is observed. Therefore, there may be some other factor(s) contributing to the increase in energy for isochorismate such as different populations of the two major conformers (pseudodiaxial and pseudodiequatorial).

The availability of large quantities of pure isochorismate synthase also made it possible to do an NMR experiment whereby one can "watch" the enzyme catalyzing the interconversion of chorismate and isochorismate in an NMR tube. Thus, when we obtained the NMR spectrum of chorismate, four peaks were observed in the vinyl region corresponding to the four vinylic protons labeled a–d at the top of Figure 12. Immediately after isochorismate synthase was added to the NMR tube, two new peaks at 6.80 and 6.18 ppm (arising from protons a and c from isochorismate as shown at the bottom of Figure 12) started emerging while the intensities of the other two peaks at 5.90 and 6.52 ppm began to decrease. This change continued with time up to a point after which no further change in the relative intensity of all the vinyl proton peaks was observed, indicative of establishment of an equilibrium between chorismate and isochorismate. The presence of two different NMR peaks between chorismate and isochorismate allowed an independent measurement of the equilibrium constant. K_{eq} obtained from the NMR experiment was 0.66, in good agreement with that

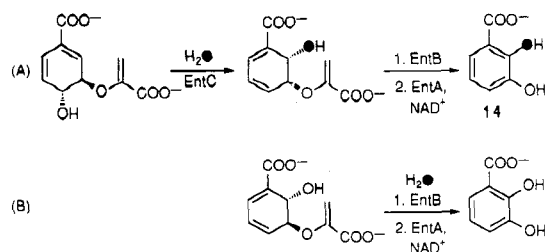


Figure 13. Oxygen-18 labeling experiment.

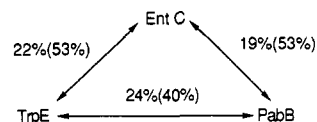


Figure 14. Three-way comparison between anthranilate synthase, isochorismate synthase, and PABA synthase with sequence similarities at amino acid level (nucleotide level) indicated.

calculated based on the kinetic analysis.

Before the detailed mechanism of this reaction was addressed, a very basic question had to be answered. That is, where is the incoming hydroxyl group from? There are conceivably two alternative sources: one from solvent water and the other from the substrate itself. To distinguish between these two alternatives, the most obvious experiment would be to conduct the reaction in H₂¹⁸O and analyze the resulting isochorismate by mass spectrometry to see whether ¹⁸O is incorporated into the product. But the unstable nature of isochorismate ($t_{1/2}$ = 6 h at 30 °C; section III.C.3) forced us to abandon this route. To circumvent this problem, we coupled the isochorismate synthase step with the two subsequent enzymes, EntB and EntA (vide infra), to convert chorismate all the way to 2,3-dihydroxybenzoate (14) (Figure 13A), a stable aromatic product that gave a strong molecular ion peak in mass spectrometry. The 2,3-dihydroxybenzoate isolated from a reaction mixture in 100% H₂¹⁸O gave a molecular ion peak with molecular weight 154. When the same reaction was repeated in a reaction with 50% H₂¹⁸O/50% H₂¹⁶O, an additional peak at m/e 156 with almost equal intensity was observed, resulting from the incorporation of ¹⁸O from the solvent. To ensure that the ¹⁸O incorporation took place at the isochorismate synthase step rather than the two subsequent conversions catalyzed by EntB and EntA, a control experiment was done in which isochorismate was used as starting material and was converted to 2,3-dihydroxybenzoate by EntB and EntA in 50% H₂¹⁸O/50% H₂¹⁶O. No incorporation of ¹⁸O was seen (Figure 13B). These results clearly established that the incoming hydroxyl group is from the solvent water with the help of EntC.

With the initial characterization of isochorismate synthase completed,⁵⁹ we have just begun to study the reaction mechanism in more detail. In what follows, we present reasonable catalytic strategies that could be used by isochorismate synthase (and anthranilate synthase and, in some cases, PABA synthase) in its first half-reaction to accelerate the double-S_N2' reaction with water (or ammonia) as a nucleophile. It may be helpful to examine structural information on these enzymes briefly. From the DNA sequences of the *E. coli* *entC*, *trpE*, and *pabB* genes and the primary sequences of the corresponding proteins, it is known that these three enzymes have about 20% homologies (Figure 14), with

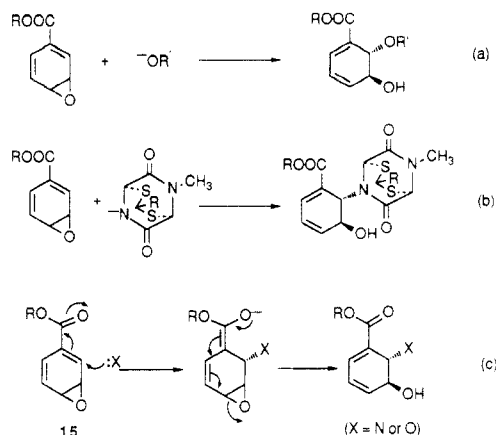


Figure 15. Chemical precedents for double- S_N2' reaction.

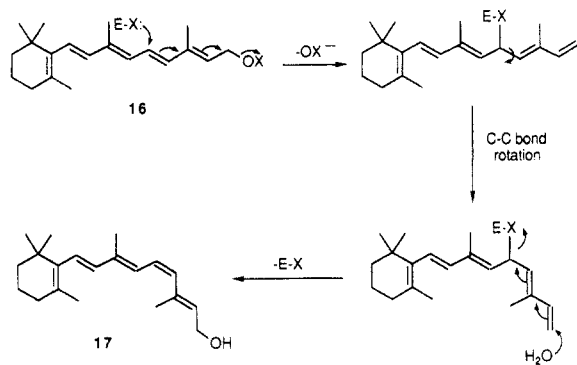


Figure 16. Biological precedent for double- S_N2' reaction.

the protein sequence identities even higher in the regions toward the carboxyl terminus.^{57,58} The homologies between these three enzymes may be attributed to the fact that a common substrate, chorismate, is used by them and consequently a similar substrate recognition domain may exist among the three proteins. However, this explanation is not as convincing considering that another chorismate-utilizing enzyme, chorismate mutase, has almost no significant homology with any of these three enzymes. Therefore, something beyond just the substrate recognition must be shared by these three enzymes, and this common feature may be related to mechanism. The premise is that these enzymes may share a common mechanism for the 1,5 double- S_N2 (and an apparent 1,1) displacement reaction.

The double- S_N2' reaction has some chemical precedent among which the most pertinent examples are perhaps those involving the benzene oxide ring opening with a nucleophile. In their synthesis of 2,3-dihydro-2,3-dihydroxybenzoate, which is the substrate for EntA, Berchtold and co-workers⁶³ accomplished the key step with an oxygen nucleophile attacking an oxepin/benzene oxide system 15 (Figure 15a). Later the same reaction was successfully employed with a nitrogen nucleophile by Fukuyama and Kishi⁶⁴ in their total synthesis of gliotoxin (Figure 15b). In both cases, the authors proposed a Michael addition followed by elimination with the cleavage of the carbon-epoxy oxygen bond (Figure 15c). This may be one of the mechanisms of enzyme-catalyzed double- S_N2' reaction as will be discussed below.

It seems that the chorismate metabolism is not the only place where nature encounters the challenge to perform a double- S_N2' reaction. The double- S_N2' displacement has recently been proposed to be involved

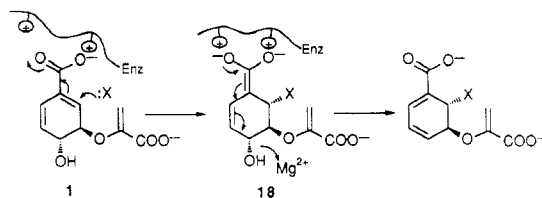


Figure 17. Michael addition/elimination mechanism.

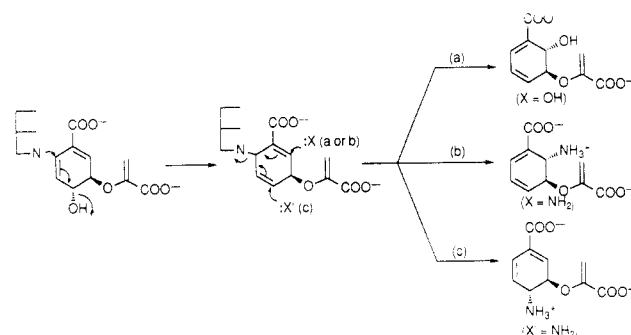


Figure 18. Covalent catalysis involving participation of a nucleophile from the enzyme active site.

in the retinal isomerase catalyzed conversion of all-*trans*-retinol (16) to 11-*cis*-retinol (17) during the visual cycle as shown in Figure 16.⁶⁵ In this case, a putative nucleophile from the enzyme active site attacks the olefin to displace the terminal OX group. After rotation of the newly formed C-C single bond, a water molecule does the reverse of the first half of the reaction, freeing the enzyme active site nucleophile in a second double- S_N2 reaction.

One unique feature of the enzyme-catalyzed double- S_N2' reaction is the absolute requirement of a divalent metal, with the most probable natural candidate being Mg^{2+} . The most obvious role Mg^{2+} may play is to chelate to the 4-hydroxyl group of chorismate, making it a better leaving group. In three out of four mechanistic possibilities proposed, we simply assume that this is the only function of the divalent metal ion although, in the fourth option, magnesium is assigned some more important roles in the catalysis as noted.

1. Michael Addition/Elimination Sequence

The Michael addition/elimination (Figure 17) is perhaps the simplest and most obvious mechanism of the enzyme-catalyzed apparent double- S_N2' reaction. In this case, the enzymes just have to stabilize the dienolate intermediate 18 by using positively charged residues. Once this dienolate is formed, a reverse Michael addition sequence expels the 4-hydroxyl group with the help of magnesium dication. This mechanism can account for the mechanisms of isochorismate synthase and anthranilate synthase but not so obviously for PABA synthase.

2. Covalent Catalysis with a Nucleophile from the Enzyme Active Site

This alternative as shown in Figure 18 is well preceded in other classes of enzymes such as the serine and cysteine protease families.⁶⁶ In this mechanism the double- S_N2' displacement is split into two consecutive single- S_N2' steps with the participation of an enzyme active site nucleophile. The major advantage of this mechanistic scheme is that the putative intermediate

CHART III

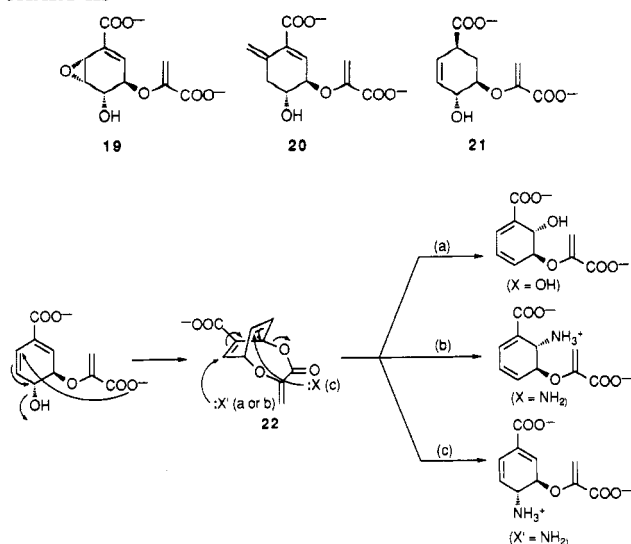


Figure 19. Bicyclic lactone as an intermediate.

is pseudosymmetric and it can lead to all three products involved in isochorismate, anthranilate, and PABA formation, accommodating all three enzymic catalyses. Thus, a second S_N2' displacement by OH at C₆ will give isochorismate (path a) and by NH₃ can yield the 6-amino-6-deoxyisochorismate intermediate (path b) in anthranilate synthase catalysis while attack at C₄ by NH₃ can lead to the 4-amino-4-deoxychorismate (path c), the intermediate⁴⁶ for PABA synthase.

In our preliminary assessment of this mechanism with anthranilate synthase and PABA synthase, compounds 19–21 (Chart III) were synthesized to serve as possible mechanism-based inhibitors to capture the putative nucleophile from the enzyme active site.⁴² Unfortunately, none of them gave time-dependent inhibition of either AS or PABAS. The design and synthesis of new inhibitors to test this mechanism are in progress.

3. Covalent Catalysis with a Nucleophile from the Substrate Itself: The Carboxylate in the Enol Pyruvyl Side Chain

Instead of using a nucleophile from the enzyme active site, the carboxylate from the C₅ enolpyruvyl side chain could also displace the 4-hydroxyl group in an intramolecular S_N2' reaction to form the putative bicyclic lactone intermediate 22 (Figure 19). A second S_N2' displacement of this lactone with water or ammonia will lead to isochorismate, anthranilate, and PABA in a fashion similar to the previous mechanism. This proposal can be tested easily when the putative bicyclic lactone 22 is synthesized.

4. Concerted Mechanism Involving a Magnesium-Bound Transition State

In all three aforementioned mechanistic possibilities, the role of the divalent magnesium was assumed to be a general acid to make the 4-hydroxyl group a better leaving group. The fact that Mg²⁺ can form up to six-coordinate complexes with oxygen ligands⁶⁷ makes it imaginable that magnesium may play some more central coordinative roles in the catalysis. For example, it may also help to deliver the incoming hydroxyl group while taking off the 4-hydroxy group in chorismate. It follows that a symmetric transition state 23 as shown

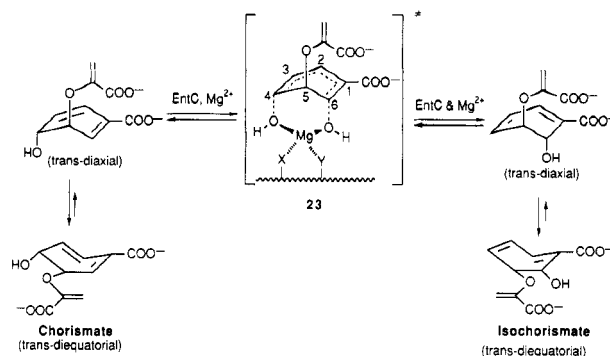
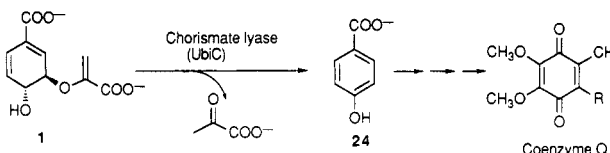
Figure 20. Concerted 1,5 double- S_N2' displacement mechanism.

Figure 21. Chorismate lyase in the biosynthesis of coenzyme Q.

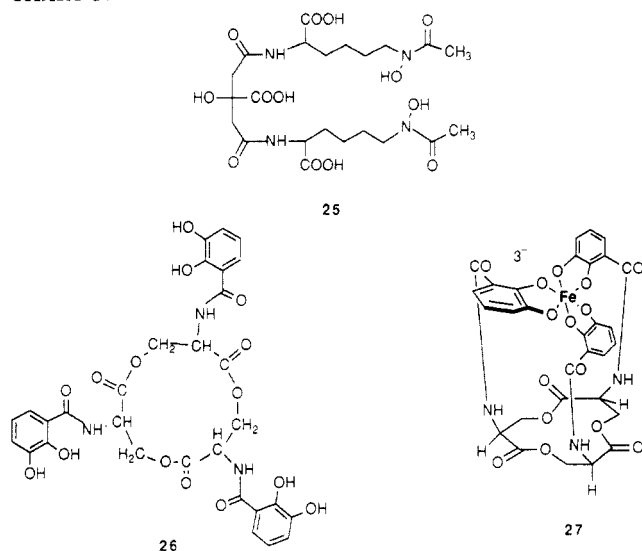
in Figure 20 may exist in the catalyses of both isochorismate synthase and anthranilate synthase. In this mechanism, the magnesium ion is bound to the enzyme with at least one water molecule in its coordination sphere. Stereoelectronic principles⁶⁸ dictate that the 4-hydroxyl group in chorismate has to be in the quasi-axial position during the displacement; hence, the trans-diaxial conformer of chorismate would be used by the enzymes. This 4-hydroxyl group can serve as a ligand for the enzyme-bound magnesium. In the transition state, the C₄-O bond is partially broken while a C₆-O bond is partially formed, with concomitant shifting of the diene π -bonds within the six-membered ring. There are two noticeable features about this transition state. One is that C₄-C₅-C₆-O-Mg-O forms a chairlike six-membered chelate ring system. The second feature is the pseudosymmetry of the transition state, which can explain the ready reversibility of the isochorismate synthase catalyzed reaction. In the case of anthranilate synthase, the magnesium-bound water can be replaced with ammonia, which is known to be a ligand, albeit weak, for magnesium and it will give rise to the aminoisochorismate intermediate for the formation of anthranilate.⁴⁰ Based on this mechanism, a series of transition-state analogues can be made giving some litmus tests for this proposal.

So far, we have not yet obtained sufficient evidence to support or reject any of the above mechanistic possibilities. Only after some of the mechanism-based inhibitors (b), putative intermediates (c), and transition-state analogues (d) are made can we gain more insight into the mechanism of these reactions.

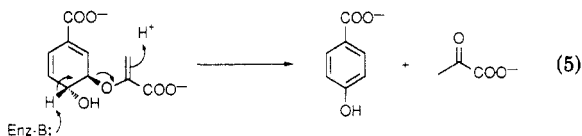
E. Chorismate Lyase

Very little work has been reported on this chorismate aromatizing enzyme that catalyzes elimination of the enolpyruvyl side chain in chorismate to yield *p*-hydroxybenzoate (24). It is known that the *ubiC* gene, encoding chorismate lyase, functions at the start of coenzyme Q biosynthesis (Figure 21), and a mutant strain of *E. coli* defective in *ubiC* gene has been isolated.⁶⁹ It seems likely that the precedents for the aromatization step by 1,2-elimination of the elements

CHART IV



of pyruvate from 2-aminoisochorismate in anthranilate synthase and 4-aminochorismate in the putative enzyme X component in PABA synthase will be relevant. Thus, an initiating abstraction of the C₄-H of chorismate by



chorismate lyase should be followed by loss of the C₃-enolpyruvyl group to yield *p*-hydroxybenzoate directly. Isolation and characterization of this enzyme should provide important information on the mechanism of the second aromatization steps in both anthranilate synthase and PABA synthase.

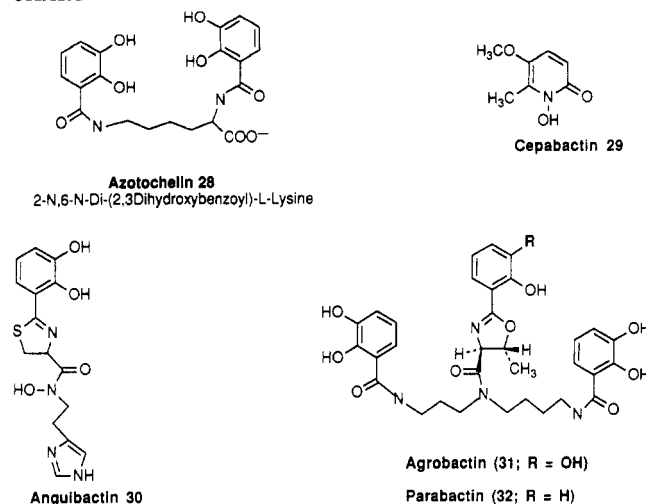
III. Mechanistic Studies in the Biosynthesis of Enterobactin

A. Introduction to Siderophores: Enterobactin

In this section we switch directions and focus on a metabolic offspring of chorismic acid, i.e., the siderophore enterobactin whose three 2,3-dihydroxybenzoate molecules are derived from this parent of many aromatic compounds. First introduce the reader to some general properties of siderophores and then specifically discuss the intricate metabolic machinery employed by *E. coli* to use enterobactin under iron-drought conditions.

Under conditions of iron scarcity, many organisms synthesize potent iron-chelating compounds termed siderophores that are secreted from the cell to sequester the available iron (as ferric iron) for uptake back into the organism where it is provided for essential metabolic processes. It is useful to classify siderophores into one of two groups, depending upon whether they contain as metal ligands a hydroxamate functionality or a phenol/catechol group.⁷⁰⁻⁷² Two siderophores that offer examples of these types are aerobactin and enterobactin (sometimes called enterochelin), both produced in *E. coli*.⁷⁰⁻⁷² In aerobactin the ferric iron is in an octahedral environment with ligands provided by the hydroxyl and free carboxylate groups of citrate and two hydroxamate groups derived from L-lysine by N-hydroxylation and

CHART V



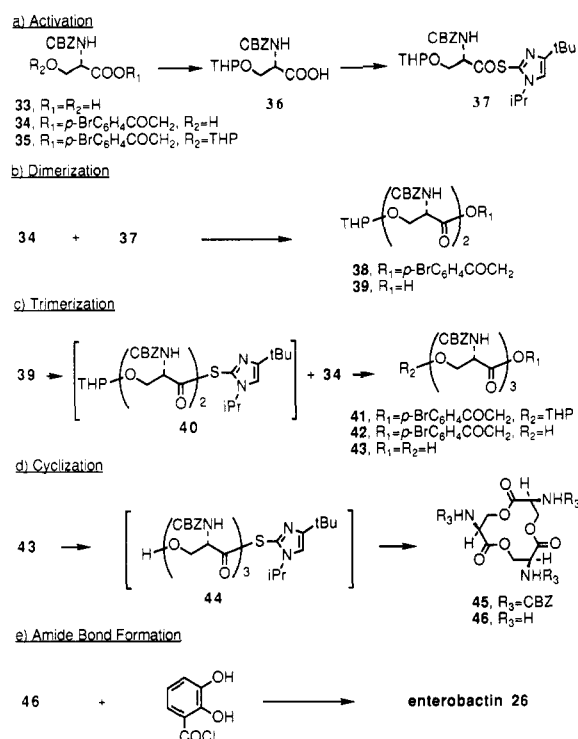
N-acetylation of the ϵ -amino group (25; Chart IV). The ligands of enterobactin derive from the catecholic groups of three 2,3-dihydroxybenzoate molecules that are linked via amide bonds to a serine trilactone cyclic scaffolding; the structure of enterobactin (26) and its ferric complex 27 are also shown in Chart IV. The ligation provided by siderophores is not limited exclusively to either phenolate or hydroxamate groups, and many siderophores use in addition carboxyl and hydroxyl oxygens (as in the aerobactin complex mentioned above), imidazole nitrogen, thiazoline nitrogen, or oxazoline nitrogen.⁷⁰⁻⁷³ Examples of siderophores (28-32) utilizing these various ligation chemistries are shown in Chart V. Interestingly, a recently discovered siderophore not falling into either class distinguished above is rhizobactin, an opine-related compound containing an ethylenediamine group.⁷⁴ For a more detailed compilation of the structure and classification of siderophores, the reader is referred to several excellent reviews.⁷⁰⁻⁷³

1. Chemical Properties of Enterobactin

Enterobactin, as shown in Chart IV, is a macrocycle composed of three units of 2,3-dihydroxybenzoylserine joined together in a cyclic structure by lactone linkages. The (proton-independent) formation constant with ferric iron of 10^{52} M^{-1} is the highest ever reported for a siderophore.⁷⁵ The redox potential of the iron complex of enterobactin exhibits a pH dependence; the potential of the ferric/ferrous couple is -0.73 V (vs NHE) at pH 7.0 whereas -0.96 V is obtained in the high-pH limit and this dependence is attributed to protonation of the ferrous enterobactin complex.⁷⁶ Since the first chelate protonation constant for the ferric complex has been measured to be $10^{4.9}$, the ferric complex of enterobactin exists largely as the $[\text{Fe}(\text{ent})]^{3-}$ form for pH ≥ 6 with the ferric ion ligated in the catechol mode.^{75,76} As the pH is lowered below 6 the *m*-hydroxyl oxygens become successively protonated with a shift in ligation to the salicylate mode, in which the ferric ion is now ligated by the *o*-hydroxyl of the catechol and the carbonyl oxygen of the amide group.⁷⁵⁻⁷⁷

The presence of three chiral centers in the molecule (each of the three serine α -carbons) leads to several possible stereoisomers, and it has been shown by Neillands et al. that only the Δ -cis isomer of the ferric

SCHEME I



chelate is active metabolically in providing iron to the organism, and not its Δ enantiomer chemically synthesized with three D-serines.⁷⁸

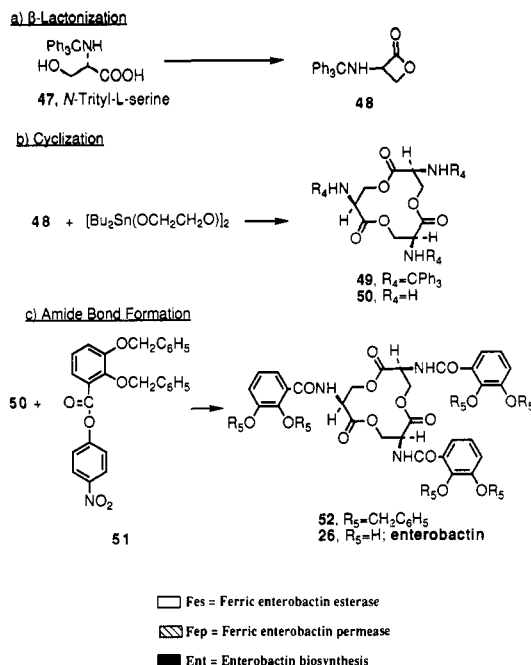
2. Synthetic Strategies for the Macrocycle Enterobactin




The first total synthesis of enterobactin was achieved by Corey and Bhattacharyya in 1977.⁷⁹ This method relies on the stepwise condensation of serine derivatives to a linear trimer and its subsequent cyclization; this cyclization is the most critical step in the synthesis of enterobactin, and a yield of 40% was obtained for this step. Corey's method is divided into five stages, diagrammed in Scheme I: (a) activation of *N*-(benzyloxycarbonyl)-L-serine (**33**), carried out in four steps (in the last step, *N,O*-protected L-serine derivative **36** was treated with 2,2'-(4-*tert*-butyl-1-isopropylimidazolyl) disulfide to give active thioester **37**); (b) dimerization by the coupling of thioester **37** with hydroxyl ester **34**; (c) trimerization by the coupling of thioester **40** with the hydroxyl ester **34**; (d) cyclization of linear trimer **43** via corresponding thioester **44** under dilute conditions; (e) amide bond formation by the reaction of cyclic triester **46** with 2,3-dihydroxybenzoyl chloride to give enterobactin (**26**). This synthetic strategy required a total of 13 steps.

The total synthesis of enterobactin has also been achieved by Rastetter et al.,⁸⁰ using a synthetic strategy very similar to the stepwise method described above. This strategy differed from Corey's method in that they used DCC/HOBt as a coupling reagent, and for amide bond formation, they used protected 2,3-bis(benzyl-oxy)benzoyl chloride instead of 2,3-dihydroxybenzoyl chloride. The use of these more efficient methods gave better yields for many of the initial coupling steps although the cyclization step gave a more disappointing result.

In an attempt to devise a more efficient synthesis, Shanzer and Libman developed a new method for the

SCHEME II



 Fes = Ferric enterobactin esterase
 Fep = Ferric enterobactin permease
 Ent = Enterobactin biosynthesis

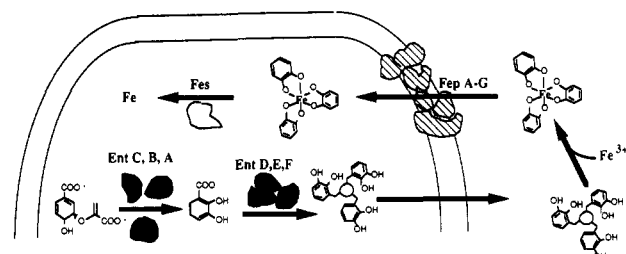


Figure 22. Components for enterobactin utilization in *E. coli*. The proteins required for the synthesis (Ent), transport (Fep), and utilization (Fes) of enterobactin are shown.

cyclooligomerization of β -hydroxy acids.⁸¹ This method is based on the use of a cyclic tin-oxygen compound as a template in the self-condensation of β -lactones to macrocyclic polylactones. Shanzer's method (Scheme II) is divided into three stages: (a) β -lactonization of *N*-trityl-L-serine (47); (b) cyclization and condensation of β -lactone 48 by treatment with the stannoxane to form the enterobactin skeleton 49 after removal of other oligomers by column chromatography; (c) amide bond formation by reaction of cyclic triester 50 with 2,3-bis(benzyloxy)benzoic acid *p*-nitrophenyl ester as active ester followed by a reductive cleavage of the benzyl groups to give enterobactin (26). This method as applied to the synthesis of enterobactin has only four steps with an overall yield of ca. 6%.

Since the biosynthesis of enterobactin from 2,3-dihydroxybenzoate, serine, and ATP is not completely understood, it is not clear whether any of these synthetic strategies are biomimetic. In all of these syntheses, the ester bonds were formed initially, followed by cyclization, and the last step was amide bond formation. From available evidence, these steps may be reversed in the biological system (section III.D).

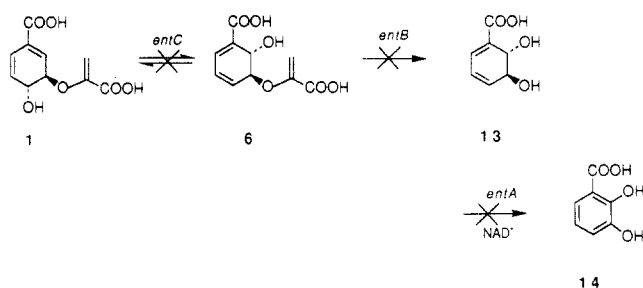
3. Metabolism of Enterobactin in *Escherichia coli*

In *E. coli* the machinery required for enterobactin metabolism can be divided into components for (1) biosynthesis and export of enterobactin, (2) transport of the ferric chelate back into the cell, and (3) processing

TABLE I. Properties of the Proteins Involved in Enterobactin Metabolism in *E. coli*

gene	protein	function	MW	location
<i>entA</i>	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (EntA)	NAD ⁺ -dependent oxidn of 2,3-diHDB to 2,3-dihydroxybenzoate	26 249	cytoplasm
<i>entB</i>	isochorismatase (EntB)	enolpyruvyl transferase enol ether hydrolysis	32 554	cytoplasm
<i>entC</i>	isochorismate synthase (EntC)	1,5-addn/elim	42 917	cytoplasm
<i>entD</i>	EntD	component of enterobactin synthase	23 579	inner membrane
<i>entE</i>	2,3-dihydroxybenzoate-AMP ligase (EntE)	synthesis of (2,3-dihydroxybenzoyl)adenylate	59 299	cytoplasm
<i>entF</i>	EntF	component of enterobactin synthase-synthesis scaffold	160 000	cytoplasm
<i>entG</i>	EntB	component of enterobactin synthase	32 554	cytoplasm
<i>fepA</i>	FepA	transport of ferric enterobactin	79 908	outer membrane
<i>fepB</i>	FepB	transport of ferric enterobactin	32 000–37 000	periplasm
<i>fepC</i>	FepC	transport of ferric enterobactin	31 000	inner membrane
<i>fepD</i>	FepD	transport of ferric enterobactin	?	inner membrane
<i>fepE</i>	FepE	transport of ferric enterobactin	43 000	membrane
<i>fepG</i>	FepG	transport of ferric enterobactin	?	inner membrane
<i>fes</i>	ferric enterochelin esterase (Fes)	hydrolysis of enterobactin lactone bonds	42 573	cytoplasm

SCHEME III



of the ferric chelate inside the cell for release of iron.⁸² Figure 22 diagrams the organization of these components as they are currently known in *E. coli*, and Table I summarizes some of the relevant properties of each of these components.

a. Biosynthesis of Enterobactin: The *ent* Genes.

Enterobactin was first discovered in culture supernatants of *Salmonella typhimurium* grown under low-iron growth conditions⁸³ and also isolated under similar environmental conditions from *E. coli* and *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*).⁸⁴ Shortly thereafter, portions of the biosynthetic pathway in *E. coli* were elucidated by Gibson et al. when six distinct mutants that grew poorly in low iron media were isolated.^{85,86} Three of these mutants, designated *entA*, *entB*, and *entC*, could grow in low-iron media when supplemented with 2,3-dihydroxybenzoate (14), a compound derived from chorismic acid (1), and were shown to be defective in its biosynthesis (Scheme III). The *entC* mutation was shown to be a defect in the enzyme isochorismate synthase, which is the first step in the pathway that converts chorismic acid (1) to isochorismic acid (6) and has already been discussed in section II.D. The *entB* mutation was a defect in the second enzyme which produces 2,3-dihydro-2,3-dihydroxybenzoate (13) by hydrolytic cleavage of the enolpyruvyl group of isochorismate, while *entA* mutants could not carry out the final NAD⁺-dependent dehydrogenation/aromatization step to produce the product 2,3-dihydroxybenzoate (14).⁸⁵

Three other mutations, designated *entD*, *entE*, and *entF*, were shown to be defective in the biosynthetic steps of enterobactin after the formation of 2,3-dihydroxybenzoate.⁸⁶ Interestingly, no detectable intermediates were formed when cell extracts prepared from any of these three mutants were incubated with 2,3-dihydroxybenzoate, L-serine, and ATP, and thus the

steps leading to the overall synthesis of enterobactin after 2,3-dihydroxybenzoate remained cryptic. A further mutation in enterobactin biosynthesis, *entG*, was identified with use of bacteriophage *Mu*-induced mutagenesis and postulated to affect the conversion of 2,3-dihydroxybenzoate to enterobactin.⁸⁷ The significance of the *entG* mutation will be discussed in section III.D.

b. Uptake of the Enterobactin-Ferric Chelate: The *fep* Genes. After secretion and release from the cell, enterobactin binds ferric iron and is transported back across the both the outer and inner membranes of *E. coli*. Early work showed that the enterobactin/ferric complex and colicin B shared a common receptor, an outer-membrane-associated polypeptide of molecular weight of $\approx 81\,000$, which had a genetic locus referred to as *fepA*.^{88,89} The nucleotide sequence of the *fepA* gene has since been determined and shown to encode a polypeptide of 723 amino acids plus a 22-residue leader peptide.⁹⁰ The mature protein had a molecular weight of 79 908 and was shown to have regions of homology with other *E. coli* outer-membrane-associated proteins.

An additional gene (*fepB*) was suggested when a mutant in ferricenterobactin complex transport distinct from the *fepA* mutation was isolated.⁹¹ The existence of the *fepB* gene distinct from *fepA* was conclusively demonstrated by complementation studies with bacteriophage λ .⁹² Subcloning of an 11-kilobase *EcoR*I fragment containing the *fepB* gene led to the expression of three to four polypeptides, ranging in size from 31.5 to 36.5 kDa, the largest, designated as proFepB, was found to be associated primarily with the membrane fraction whereas the mature forms of FepB were enriched in the periplasm.^{93a} This study also indicated the presence of another gene, *fepC*, which encoded a polypeptide of molecular weight of 30 500 that was also associated with the membranes and, since no proFepC protein was identified, was tentatively suggested to be an inner membrane component. The *fepB* gene has recently been sequenced; the resulting polypeptide consists of 318 residues with a predicted molecular weight of 34 255 and contains a 22-residue signal peptide.^{93b} Interestingly, the FepB protein does not contain any cysteine residues. Thus, three essential components of the ferric/enterobactin permease (Fep) complex had been identified and shown to be distributed between the outer membrane (FepA), periplasm (FepB), and the inner membrane (FepC). The recent crystalization of

the FepA protein promises to yield exciting information regarding the molecular basis for ferric enterobactin recognition.⁹⁵

Further work investigating the genetic organization of the *fep* genes using insertional mutagenesis and other genetic strategies has identified three more potential permease genes: *fepD*, the first gene in the transcript which includes *fepG* and *fepC*; *fepE*, which weakly expresses a 43.0-kDa polypeptide in minicells and is located between the *entF* and *fepC* genes; *fepG*, which is located between *fepC* and *fepD*.^{94a} An additional polypeptide identified in that study thought to be the *fepF* gene product which lies between *fepB* and *entE* has subsequently been shown in fact to be the product of the biosynthetic *entC* gene, isochlorismate synthase. Although the exact function of the FepC, FepD, and FepG proteins is not known, they have recently been found to be related to other peptides found in protein-dependant transport systems such as those found for ferric citrate and ferric aerobactin transport, the B₁₂ transport system, and the maltose transport system, with FepC encoding a potential ATP binding protein.^{94b-d}

In addition to the specific ferric/enterobactin permease components, less specific transport systems are also involved in the uptake of the ferric/enterobactin complex. The *tonB* and *exbB* genes both have pleiotropic phenotypes; in addition to their role in ferric enterobactin transport, they are also involved in the uptake of vitamin B₁₂.^{96,97}

c. Processing of the Complex for Iron Release: The *fes* Gene. After intracellular retrieval of the ferric/enterobactin complex, the iron must be released for metabolic use and there have been two mechanisms proposed to accomplish this in *E. coli*. The first of these involves a ferric/enterochelin esterase (Fes), which functions to hydrolyze the macrocycle into the monomeric (2,3-dihydroxybenzoyl)serine units, which would be expected to bind the ion less tightly. The isolation of a *fes* mutant supported this proposal.⁹⁸ However, analogues of enterobactin were synthesized that do not contain any hydrolyzable substituents, e.g., 1,3,5-tris[[N,N',N''-tris(2,3-dihydroxybenzoyl)amino]-methyl]benzene (MECAM), which are able to support growth of *ent*⁻ strains under low-iron conditions.^{99,100} This intriguing result led to the proposal that a reductase may be involved that reduces the iron to the ferrous state, which is known to have a lower binding constant with enterobactin.⁷⁶ The difficulty of reducing the ferric/enterobactin complex (section III.A.1) was explained by Raymond as possibly occurring in the lower pH environment of the periplasm where the potential for reduction could be achieved by physiological reductants.^{76,99,100} Recently, the Fes protein has been subcloned, expressed, purified, and shown to hydrolyze both enterobactin and its ferric chelate.¹⁰¹ The physiological route by which iron is released from enterobactin, however, is still uncertain.

B. Genetic Organization and Regulation of Enterobactin Metabolism

By a variety of molecular biological techniques and cloning strategies, the known genes necessary for enterobactin synthesis (*ent*), transport (*fep*), and processing (*fes*) have been located and shown to span ap-

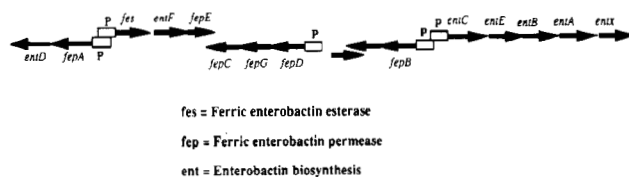


Figure 23. Gene cluster of the *ent*, *fep*, and *fes* genes. The genes for enterobactin utilization are clustered into two bidirectional and one unidirectional iron-regulated control units under the control of promoters and iron-dependent regulatory elements (iron boxes). The *ent*, *fep*, and *fes* genes span approximately 22 kilobases of DNA.

proximately 22 kilobases of the *E. coli* chromosome (Figure 23) as two bidirectional and one unidirectional iron-regulated control units.^{53-55,102-107} The bidirectional units are each composed of two operons that are transcribed in opposite directions, and each under tight transcriptional control by iron and the DNA binding Fur protein.^{57,58,103,106,107}

The DNA sequence encompassing the *ent*, *fep*, and *fes* genes has now been determined, and the location of these 14 genes has been verified. Thus, the right-most operon contains five open reading frames, four of which correspond to the *entCEBA* genes.^{56-58,108,109} An additional open reading frame, *entX*, which codes for a polypeptide of molecular weight 14 970 and has as yet unidentified and presumably nonessential physiological role, is located downstream of *entA*. The original designation of *entC*⁵⁵ has since been shown to be the *entA* gene;¹¹⁰ the correct location of the *entC* gene has now been determined to be the first gene in the *entC-EBA* operon.^{57,58} The DNA sequence has also been reported for the region containing the *fes* gene and part of the 5'-region of the *entF* gene.^{106,107} This region also contains a small open reading frame of a polypeptide of 72 amino acids with an unknown function. Recently, the sequence of the *fepA* and *entD* genes has been determined,^{90,111a,b} completing the sequence of the left-most operon of the enterobactin cluster after the *fepA* gene. The DNA sequence of the rest of the *fep* genes and also the rest of the *entF* gene is also now known.^{93b,94b,c,165}

Perhaps one of the most interesting features of the enterobactin cluster of genes is transcriptional regulation by intracellular iron concentrations, mediated by the Fur protein.^{57,58,106,107} The first indications of iron-regulated systems occurred with the isolation of *fur* mutants, which constitutively expressed iron-regulated proteins.¹¹² The *fur* gene has since been cloned by complementation screening;¹¹³ sequence analysis indicated that *fur* encodes a small polypeptide of 16.8 kDa.¹¹⁴ Neilands' group has shown that purified Fur protein acts as a repressor, requiring as a corepressor a divalent metal ion (in vivo, Fe²⁺). As illustrated in Figure 24, the metal/Fur complex binds to a palindromic DNA sequence that has been identified as an "iron box" and prevents transcription.^{115-118,119a,b} The Fur protein has since been shown to be involved in the iron-dependent regulation of a number of genes in *E. coli* besides those necessary for enterobactin utilization (Table II).¹²⁰⁻¹²⁴

In the *E. coli* enterobactin cluster of genes, the DNA sequence of the regions between the *entC* and *fepB* genes (≈250 base pairs) and the *fepA* and *fes* genes has been shown to encode both promoter and regulatory elements; each of these regions contain elements of two

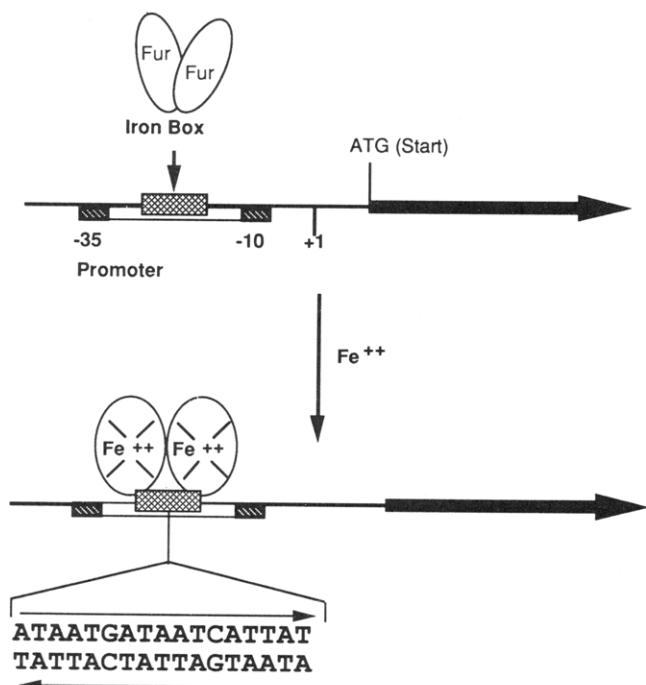


Figure 24. Fur protein as a repressor, requiring Fe^{2+} as corepressor. The Ferric uptake repressor (Fur) protein binds to the iron box region within the promoter region to repress transcription. Repression requires a divalent metal ion such as Fe^{2+} . The consensus palindromic iron box is shown.

TABLE II. Some *E. coli* Genes Regulated by Iron and the Fur Protein

gene	protein	ref
<i>entA-F</i>	enterobactin biosynthesis	58
<i>fepA-G</i>	enterobactin transport	107
<i>incA-D (aerA-D)</i>	aerobactin biosynthesis	120
<i>fhuA-E iutA</i>	aerobactin transport	
<i>sltA, sltB</i>	shiga-like toxin	121, 122
<i>cirA</i>	colicin I receptor	123
<i>sodA, sodB</i>	Fe and Mn superoxide dismutase	124a,b

promoters, each controlling the transcription of genes in the opposite direction.^{57,58,106,107} These regions also contain two putative opposing "iron boxes" based on the consensus sequence for these regulatory elements. An additional "iron box" has been found preceeding the *fepD* gene.^{93b,94b} Figure 25 indicates the organization of the bidirectional iron-regulated regions of the *ent*, *fep*, and *fes* gene cluster and shows that the iron boxes are situated within the promoter regions. Interestingly, in the *fepA-fes* promoter region, both bidirectional promoters and iron boxes are overlapping on opposite strands of the DNA.

C. Biosynthesis of the 2,3-Dihydroxybenzoate Moiety of Enterobactin

From studies using *E. coli* mutants it is known that 2,3-dihydroxybenzoate (14) is derived from chorismate (1) by the action of three enzymes: isochorismate synthase, isochorismatase, and 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (2,3-diDHB dehydrogenase), often called by their genomic mnemonics EntC, EntB, and EntA (Scheme III). Mechanistically, each of these three enzymes is intriguing since they catalyze rather unusual reactions. Isochorismate synthase catalyzes a net 1,5-addition/elimination reaction directly analogous to the reaction carried out by an-

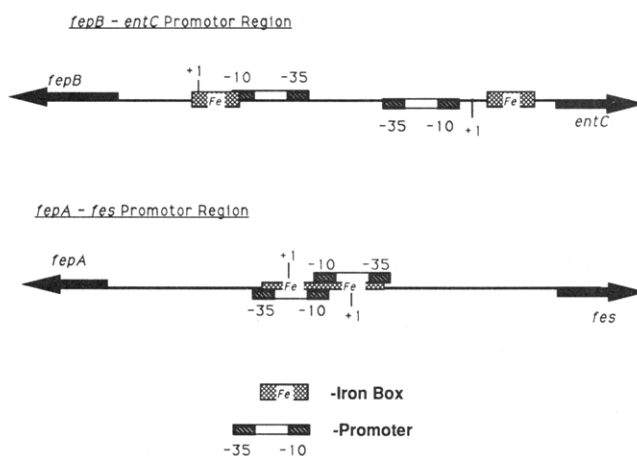


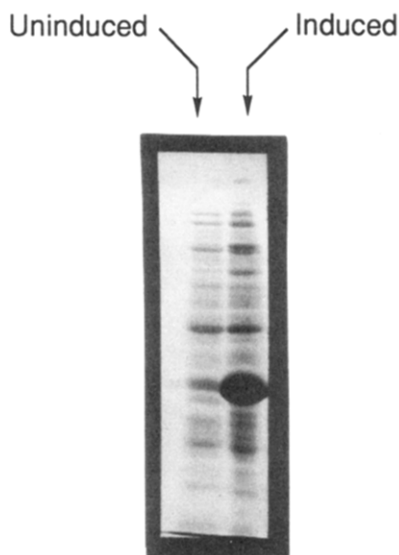
Figure 25. Regulatory regions of the *ent* gene cluster. Both the *fepB-entC* and the *fepA-fes* regions contain promoter and iron-dependent regulatory elements. The -10 and -35 regions of the promoter as well as the iron boxes are indicated. In the *fepA-fes* region, the promoters and iron boxes are overlapping on both strands of the DNA, directing transcription in opposite directions.

thranilate synthase without subsequent aromatization. Insight into the mechanistic details for the isochorismate synthase reaction has already been forwarded in section II.D. The reaction catalyzed by isochorismatase, although a seemingly simple hydrolysis liberating pyruvate and 2,3-dihydro-2,3-dihydroxybenzoate (13), is unique in that the substrate for the reaction is an enol ether. Finally, a 2,3-diDHB dehydrogenase functions as a straightforward alcohol dehydrogenase, but the product of the reaction, an unstable cyclohexadienone, rapidly rearranges to the more stable aromatic catechol.

1. Molecular Biological Strategy

In order to study the mechanistic details of each of these enzymes, substantial quantities of each protein were required and purification from the wild-type sources would not provide sufficient material. We turned then to the tools of modern molecular biology to overexpress these proteins, which provided a 2-fold advantage: (1) with the overexpression systems employed to overproduce the EntC, EntB, EntA, and EntE proteins, a range of 1–50 mg of protein/L of culture has been obtained in these systems, supplying ample protein for mechanistic studies; (2) with the overexpressed protein amounting from a few percent to over 50% of the total cellular protein, the purification protocol is simplified substantially over a procedure for wild-type levels of enzyme.

The protocol, then, involves four steps, the first of which involves cloning the genes for the sought-after enzymes and is probably the most time-consuming step involved. Fortunately, this had already been accomplished for the *ent* genes by the laboratories of Dr. Mark McIntosh at the University of Missouri—Columbia, Dr. Charles Earhart from the University of Texas at Austin, and Dr. Ian Young from the Australian National University, who provided us with plasmids encoding these genes. For the *ent* genes, cloning was accomplished by selection procedures that could detect complementation of mutant phenotypes. Alternative strategies for cloning such as the use of synthetic oligonucleotides based upon the N- and C-terminal peptide sequence of the protein of interest requires prior



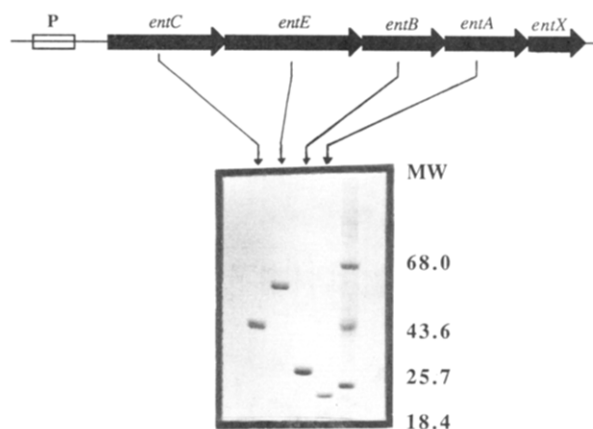
- 55% of soluble cell protein
- 90 mg of pure enzyme/liter of cell culture

Figure 26. SDS-PAGE gel of an overproducing construct for the *entB* gene. On the left are whole-cell extracts under uninduced conditions, while on the right the overexpression of the Ent B Protein is obvious after the inducer isopropyl β -D-thiogalactoside is added. Over 55% of the total cell protein is accounted for by EntB with this particular construct, yielding 90 mg of pure enzyme from 1 L of cell culture.

purification of the enzyme. Although sequencing is not absolutely required to express the protein once the gene has been cloned, it is recommended since sequence data can provide information such as restriction sites flanking and within the gene and regulatory elements in the DNA sequence on the 5'-end of the gene such as promoters and ribosome binding sites. This information is often critical for obtaining good expression since the promoter affects the level of transcription of the gene whereas the ribosome binding site and the sequences between the ribosome binding site and the start codon of the gene influence the efficiency of protein translation.

In the third step, following cloning (and sequencing), the gene is next subcloned into a multicopy expression vector that utilizes strong inducible promoters to direct the transcription of the inserted gene. We have had good success using the hybrid *tac* promoter in the vector pKK223-3, or the system developed by Tabor and Richardson using the bacteriophage T7 promoter.¹²⁵ As a case in point, using a procedure to delete 800 bases of unnecessary DNA upstream of the *entB* gene followed by subcloning the resulting *entB* fragment into pKK223-3 resulted in an exemplary overexpression system that overproduced isochorismatase to >50% of the cellular protein. From 1 L of culture, we can obtain 80–90 mg of homogeneous enzyme after a simple purification protocol (Figure 26).¹²⁶

The last step involves purification of the protein of interest. Using the tenets of this strategy, we have successfully purified the proteins encoded for by the genes of the right-most operon of Figure 23 in amounts suitable for mechanistic studies. Thus, Figure 27 displays a denaturing acrylamide gel run with purified



Predicted Molecular Weights from Gene Sequence

EntC:	42,917
EntE:	59,299
EntB:	32,554
EntA:	26,249

Figure 27. SDS-PAGE gel of the purified Ent C, Ent E, Ent B, and Ent A proteins. The molecular weight of the purified proteins determined from the PAGE gel matches closely the size predicted from the gene sequence.

samples of isochorismate synthase (EntC), 2,3-dihydroxybenzoate-AMP ligase (EntE), isochorismatase (EntB), and 2,3-diDHB dehydrogenase (EntA) obtained in this manner. We now turn to a discussion of each of these proteins.

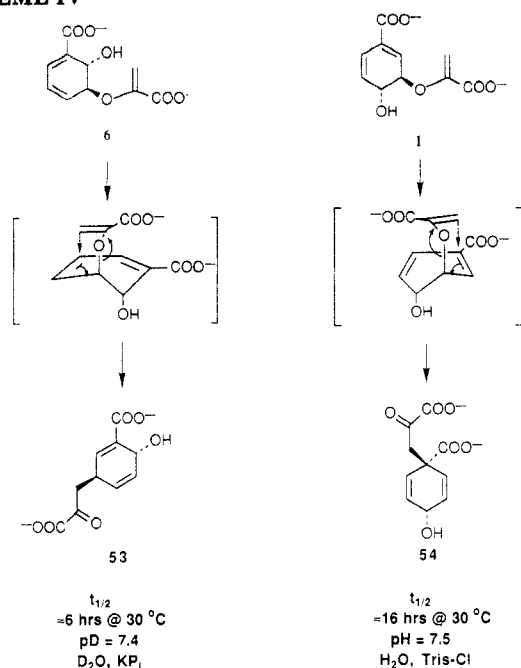
2. Isochorismate Synthase

Isochorismate synthase (E.C. 5.4.99.6), the first enzyme involved in the biosynthesis of 2,3-dihydroxybenzoate in the biosynthesis of enterobactin, has already been discussed in section II.D. The purification of isochorismate synthase⁵⁹ and the proceeding enzyme in the pathway to 2,3-dihydroxybenzoate, isochorismatase,¹²⁶ represent the first enzymes purified to homogeneity that utilize isochorismic acid as a substrate.

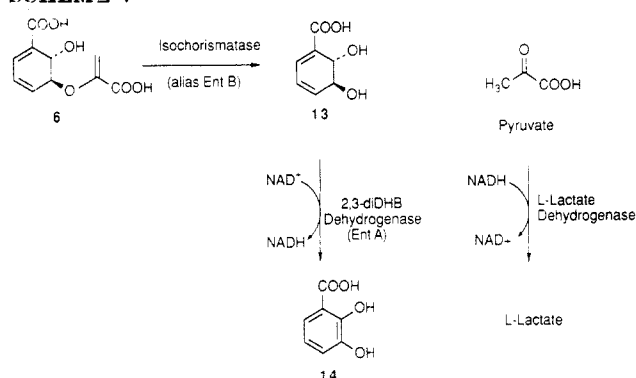
3. Chemical Properties of Isochorismic Acid

Isochorismic acid (**6**), the chemical cousin of chorismic acid, was first characterized as an intermediate in the enterobactin biosynthetic pathway and was shown to be a rather unstable compound that decomposed at room temperature and neutral pH to salicylate and 3-(carboxyphenyl)pyruvate.⁵⁰ The structure was proposed to be *trans*-5-[(1-carboxyethenyl)oxy]-6-hydroxy-1,3-cyclohexadiene-1-carboxylic acid, and the absolute stereochemistry was determined to be 5*S*,6*S* at the carbon positions bearing the hydroxyl and enolpyruvyl groups.⁵⁰ The first total synthesis of isochorismic acid, accomplished by Busch and Berchtold,¹²⁷ confirmed the initial structural determination. The ultraviolet absorption spectrum of isochorismate in water shows a strong absorbance at 278–280 nm with

SCHEME IV



SCHEME V



an extinction coefficient of $12.2\text{--}13.0\text{ mM}^{-1}\text{ cm}^{-1}$,⁵⁰ although a much lower extinction coefficient of $8.3\text{ mM}^{-1}\text{ cm}^{-1}$ has recently been forwarded.¹²⁶ The higher extinction coefficient of isochorismic acid versus chorismic acid ($\epsilon_{275} = 2.6\text{ mM}^{-1}\text{ cm}^{-1}$ ¹²⁸) is consistent with extended conjugation of the diene system with the carboxylate group of isochorismic acid. Using an NMR analysis to quantify the decomposition products of isochorismate, Berchtold was able to demonstrate that the major breakdown route at 30 °C in water was via concerted 3,3-rearrangement to isoprephenate (53)¹²⁹ and was almost 3 times faster than the corresponding rearrangement of chorismate to prephenate (54) (Scheme IV).¹³⁰

4. Isochorismatase

The second enzyme in the biosynthesis of 2,3-dihydroxybenzoate, isochorismatase (E.C. 3.3.2.1), catalyzes the hydrolytic cleavage of the enolpyruvyl side chain of isochorismic acid (6) to yield 2,3-dihydro-2,3-dihydroxybenzoate (13) and pyruvate (Scheme V). First identified in crude extracts,^{50,51} the enzyme has recently been purified to homogeneity from an over-producing strain of *E. coli* by a continuous photometric assay diagrammed in Scheme V.¹²⁶

Analogues of isochorismate were synthesized to probe the specificity of the enzymatic reaction (Table III); the

TABLE III. Substrate Specificity of Isochorismatase

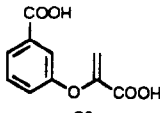
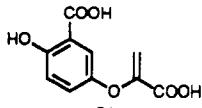
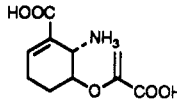
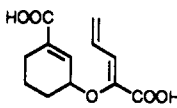
	no.	$K_m(\mu\text{M})$	$k_{\text{cat}}(\text{min}^{-1})$	rel k_{cat}/K_m
	6	15	600	100
	55	23	310	34
	56	120	540	11
	57	280	980	9
	58	86	1300	38
	1	$\geq 37\,000$	≥ 1300	0.088
	59	nd	≥ 2.3	nd

results indicated that the enzyme can tolerate a number of changes in the substrate structure. Thus, even though isochorismate (6) is the most efficient substrate in terms of k_{cat}/K_m , four other compounds 55–58 were used quite efficiently and they have a double-bond system different from that of isochorismate and, except 55, are missing the hydroxyl group.

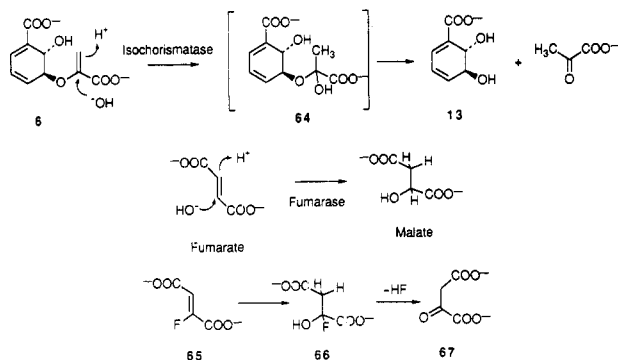
A number of other compounds with substituents at C₄ were also converted by isochorismatase, albeit at a much reduced catalytic efficiency. The most interesting of these was chorismic acid (1), the precursor to isochorismate (6) in the biosynthetic pathway. The expected hydrolysis product, 3,4-dihydro-3,4-dihydroxybenzoate, was confirmed with ¹H NMR spectroscopy.¹²⁶ This result explains an earlier observation for the formation of 3,4-dihydro-3,4-dihydroxybenzoate in crude extracts of *Aerobacter aerogenes* grown in an iron-deficient medium.¹³¹ This side reaction seems counter-productive for the efficient production of 2,3-dihydroxybenzoate (and enterobactin) although the large K_m measured for chorismate ($\approx 40\text{ mM}$) may prevent this reaction from occurring to an appreciable extent in vivo.¹²⁶ The large value of K_m for chorismate is most likely a result of the substituent at C₄ since compound 59 also exhibits a large K_m in the millimolar range (the K_m for 59 has not been measured since the rate is essentially linear up to 2 mM, thus setting a lower bound for K_m for this compound).

Cycloheptadiene 56 and heterocyclic analogue 59 are interesting examples of tolerance by the enzyme for the ring structure of the substrate. The fact that aromatic compounds 60 and 61 (Table IV) were not hydrolyzed (but were competitive inhibitors) may suggest that the enolpyruvyl side chain is unable to move into the proper orientation in the active site for these compounds. Interestingly, compound 62, the amino version of di-

TABLE IV. Inhibitors of Isochorismatase (Ent B)

compound	$K_i(\mu\text{M})$	type
 60	130	competitive
 61	2000	competitive
 62		not inhibitory
 63		not inhibitory

SCHEME VI



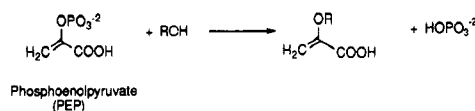
hydroisochorismate (**55**), was neither a substrate nor an inhibitor for isochorismatase, indicating that the presence of the amino group must influence the binding of this compound to the enzyme. We have recently tested compound **63**, an analogue of **58**, which has extended conjugation on the side chain, and found it to be neither a substrate (as evaluated in a coupled assay with EntA, see section III.C.5) nor an inhibitor.¹³²

Intuitively, the isochorismatase-catalyzed reaction must involve two half-reactions, the first being addition of water across the double bond of the enolpyruvyl side chain to form the transient hemiketal **64** (Scheme VI). Being unstable, this intermediate will collapse in the second half-reaction to eliminate alcohol **13** and pyruvate. The first half-reaction is analogous to hydratase-catalyzed reactions such as the fumarase-catalyzed hydration of fumarate; however, in the fumarase reaction the product is stable (malate) and does not react further. The isochorismatase reaction is more akin to the reaction of fumarase with α -fluoromalate (**65**), which has been shown to proceed via the hydrated intermediate **66** and decompose directly to α -ketoglutarate (**67**).¹³³

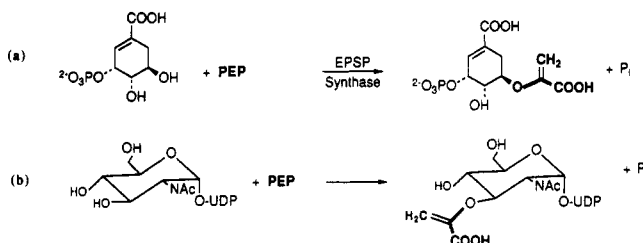
In a formal sense, isochorismatase is an enzyme in which an enol ether group is involved in an enzyme-catalyzed group-transfer reaction. We have classified these into two groups: type I enzymes catalyze the reversible transfer of enolpyruvate between phosphate and an alcohol, whereas type II bring about the group transfer of an enol ether group to water.¹²⁶ The type I enzymes function in the forward direction in metab-

SCHEME VII

Type I: Enolpyruvyl Ether Synthesis

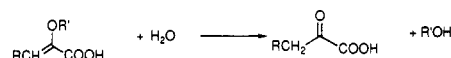


Examples:

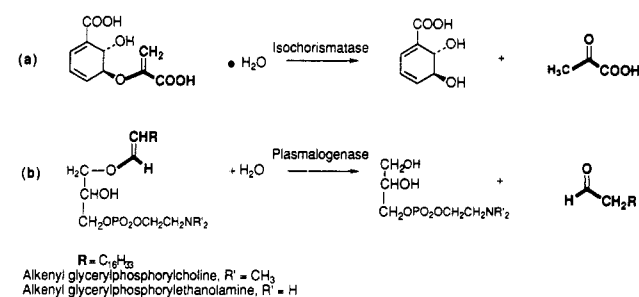


SCHEME VIII

Type II: Enolether Hydrolysis

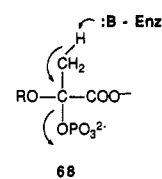


Examples:

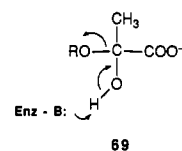


SCHEME IX

Type I

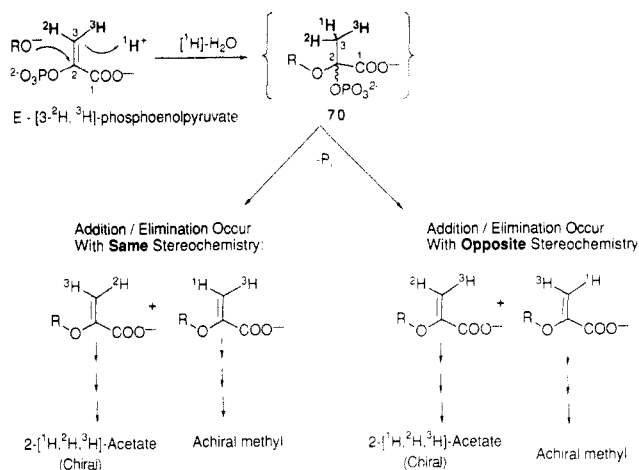


Type II



olism, utilizing phosphoenolpyruvate as the donor. Examples of the type I enzymes are shown in Scheme VII. Enolpyruvylshikimate 3-phosphate synthase (EPSP synthase) catalyzes the addition of the enolpyruvyl group to shikimate 3-phosphate in the biogenesis of chorismate, releasing inorganic phosphate in the process.¹³⁴ Another enzyme carrying out this transformation is the first enzyme in bacterial peptidoglycan biosynthesis, which adds the enolpyruvyl group to UDP-*N*-acetylglucosamine.¹³⁵⁻¹³⁷ Examples of the type II enzymes are isochorismatase and the enzyme plasmalogenase, which catalyzes the hydrolysis of alkenyl ether bonds found in plasmalogens (Scheme VIII).¹³⁸⁻¹⁴¹

SCHEME X

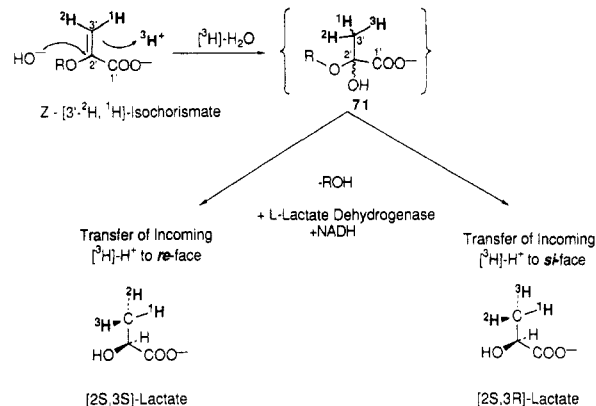


In both the type I and II enzymes, a tetrahedral intermediate is formed (Scheme IX), involving protonation to form a methyl group. In the type I enzymes, deprotonation of this intermediate (from the transiently formed methyl group) may be enzyme-assisted and can lead to elimination of either phosphate in the forward direction or alcohol in the reverse direction. Intermediate 68 in the type I enzyme-catalyzed reactions has been detected as an intermediate in the EPSP synthase reaction.¹⁴² Although a different mechanism involving an enzyme/enolpyruvate intermediate has been postulated in the reaction with enolpyruvyl UDP-*N*-acetylglucosamine transferase,¹³⁷ an intermediate such as 68 has not been rigorously ruled out. The deprotonation step in the type II enzymes 69, however, most likely occurs from the hydroxyl group (due to the lower pK_a relative to methyl) and may occur spontaneously.

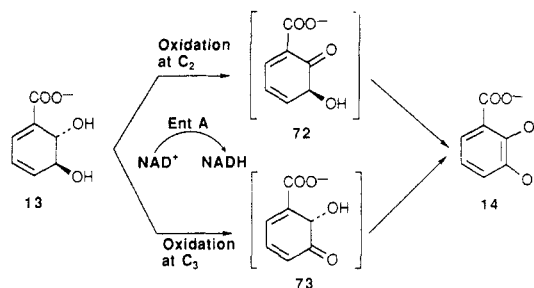
Two stereochemical questions arise for the mechanisms proposed for the type I and type II enzymes: (1) From which face does protonation/deprotonation of the methyl group occur? (2) Does addition across the double bond occur as syn or anti (for the type I enzymes; this question also arises for the elimination step)? These issues have been addressed for the reaction catalyzed by EPSP synthase with use of doubly isotopically labeled phosphoenolpyruvate (PEP) (e.g., (*E*)- and (*Z*)-[3-²H, ³H]phosphoenolpyruvate; Scheme X).¹⁴³⁻¹⁴⁵ When the reaction is run in ¹H₂O, the methyl group of the transient intermediate 70 contains ¹H, ²H, and ³H and will be chiral if protonation occurs stereospecifically. The elimination step will produce two species containing tritium in the methylene group, one of which can be converted into a chiral methyl group by stereospecific introduction of ¹H. This chiral methyl-containing product can then be used to determine the stereochemical course of the reaction.¹⁴³⁻¹⁴⁵ Thus, it was determined that the addition/elimination steps occurred with opposite stereochemistry; i.e., if addition occurred anti, then elimination occurred syn or vice versa. Unfortunately, information of the chirality at C₂ or C₃ or the transiently formed tetrahedral intermediate 70 (Scheme X) could not be inferred from these studies. However, using chiral phosphonate analogues of the intermediate formed in the EPSP synthase reaction, Alberg and Bartlett have suggested that tetrahedral carbon at C₂ has the *R* configuration.¹⁴⁶

Since the protonation/deprotonation steps in the type II enzymes involve different groups, the chirality

SCHEME XI



SCHEME XII



of the methyl group of intermediate 71 (Scheme XI) can be determined directly (again with use of a doubly isotopically labeled substrate such as (*Z*)-[3'-²H, ¹H]-isochorismic acid and running the reaction in ³H₂O). However, with this methodology, the stereochemistry of the addition step remains cryptic and other methods are required for this determination.

5. 2,3-Dihydro-2,3-dihydroxybenzoate Dehydrogenase

The enzyme 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (E.C. 1.3.1.28), alias EntA, catalyzes the final step in the formation of 2,3-dihydroxybenzoate (14) (Scheme III) by oxidizing dihydrodiol 13 (2,3-diDHB) in an NAD⁺-dependent step.⁵⁶ It was anticipated that EntA would function as an alcohol dehydrogenase to oxidize either the C₂ or C₃ hydroxyl groups to produce the corresponding hydroxycyclohexadienone 72 or 73 (Scheme XII) as a transient intermediate that would rapidly tautomerize to aromatic catechol 14. Unfortunately, with the natural substrate, oxidation at either carbon produces the same aromatic product.

That EntA proceeds via alcohol oxidation was verified with a number of 2- and 3-deoxy and dihydro analogues of 2,3-diDHB (Table V), which determined that the regiochemistry of oxidation occurred at C₃.¹⁴⁷ Hence, only those compounds containing a hydroxyl group at C₃ (but not C₂) were oxidized by EntA. Of these, the simplest change from 13 was the deoxy analogue 74, which maintains the substrate architecture except the C₂ alcohol is missing. This compound has about the same K_m as the natural substrate 13, but it is turned over by the enzyme at about one-fifth the rate. Other compounds showing decreasing catalytic efficiency (k_{cat}/K_m) are compounds 75-79, which differ in the allylic system compared with 13. Interestingly, the minimum substrate recognition was provided by cyclohexanol (82), and thus the carboxylate functionality

TABLE V. Substrate Specificity of EntA

substrate	no.	K_m (mM)	turnover K_{cat} (min ⁻¹)	rel k_{cat}/K_m	nonsubstrate	K_i (mM), type
	13	300	5550	100		83 1400, non
	74	260	1050	22		84 3300
	75	2800	1380	2.6		85 2300
	76	1900	300	0.86		86 negative
	77	1700	180	0.59		
	78	4100	300	0.38		
	79	25200	44	0.09		
	80	168000	1000	0.03		
	81	16500	60	0.02		
	82	positive				

TABLE VI. Some Alternate Substrates and Nonsubstrates of EntA

substrate	no.	K_m (μM)	turnover k_{cat} (min ⁻¹)	k_{cat}/K_m (% of 85)	nonsubstrate	no.
	87	200	260	100		92
	88	200	33	13		93
	89	700	23	2.5		94
	90	45000	14	0.024		
	91	30500	0.4	0.001		

was not strictly required although the efficiency of oxidation of this and other decarboxy analogues 80 and 81 was 4–5 orders of magnitude lower in these compounds. Compounds 83–86, on the other hand, are inhibitors but not substrates, verifying the contention that oxidation occurs at C₃.

The EntA-catalyzed reaction with the normal substrate is irreversible due to aromatization to 2,3-dihydroxybenzoate. However, if aromatization cannot occur, such as with dihydro substrates 75, 76, 78, 79, and

82 (Table V), the reverse reaction could be monitored (as NADH consumption) with the corresponding ketone compounds. This was demonstrated with compounds 87–91 (but not 92–94), which were reduced by EntA to the corresponding alcohols (Table VI).¹⁴⁷ Of these, the best substrate was 87, which had an 8–40-fold better catalytic efficiency for reduction than 88 and 89. As was noted with the alcohols in Table V, cyclohexanone (90) and cyclohexenone (91), both lacking a carboxylate group, were turned over by the enzyme albeit at 10⁴–10⁵

SCHEME XIII

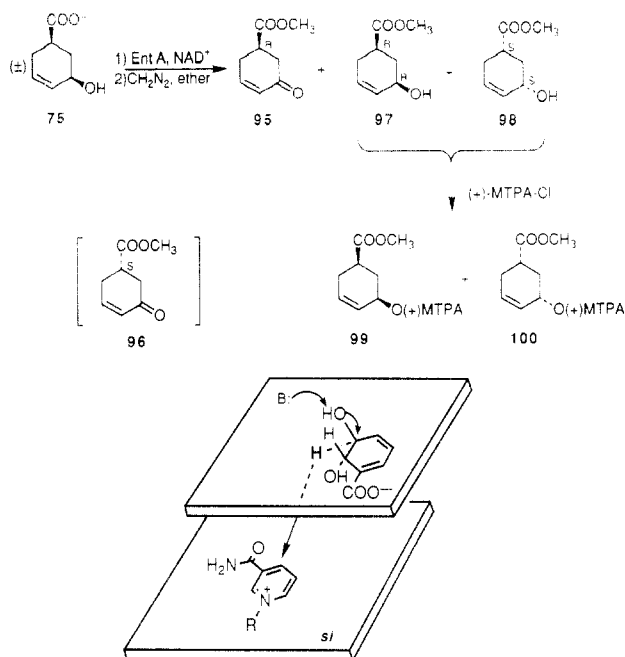


Figure 28. Schematic of the active site of the Ent A protein. From the mechanistic studies, the C₃ proton is stereospecifically removed as a hydride and transferred to the *si* face of NAD⁺.

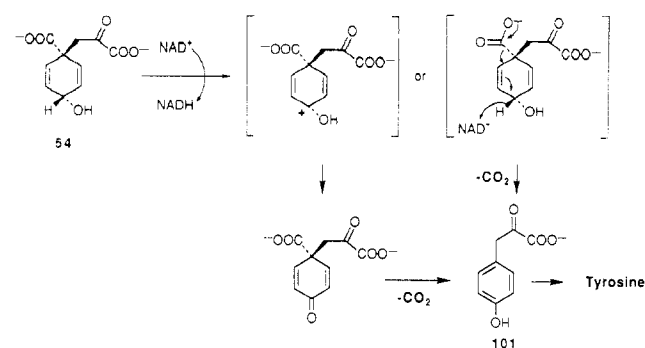
lower k_{cat}/K_m . These results not only demonstrate reversibility but also verify that the site of hydride transfer occurs at C₃.

With the regiochemistry of alcohol oxidation established, the stereochemistry of hydride extraction from substrate and transfer to coenzyme was the next mechanistic question to be addressed. Both of these processes would be expected to proceed stereospecifically. Although the absolute stereochemistry of 2,3-diHDB (13) isolated from growth cultures of iron-repressed *A. aerogenes* had been determined to be 2*S*,3*S*, stereoselectivity of the dehydrogenase had not been demonstrated.^{61,148} That EntA proceeds stereospecifically with respect to hydride transfer was indicated when oxidation of racemic alcohol 75 proceeded to less than 50% consumption (Scheme XIII). The product of the reaction (after esterification) 95 was identical in all respects to (+)-(1*S*)-96, except for the sign of rotation identifying the 1*R*,3*R* isomer as the enantiomer transformed by EntA.¹⁴⁷ Further proof was forwarded when unreacted alcohols 97 and 98 were converted into the Mosher esters 99 and 100. The ¹H NMR resonance of the diastomeric methoxy group of the Mosher ester of unreacted starting material indicated an abundance of 1*S*,3*S* isomer 100, again suggesting a preference for the 1*R*,3*R* material.

The stereospecificity of hydride transfer to NAD⁺ was then determined with use of a specifically deuterated version of 76 to produce [4-²H]NADH, the chirality of which could be determined by ¹H NMR. After incubation of deuterated alcohol 76 with EntA and NAD⁺, the [4-²H]NADH isolated was shown to have deuterium incorporated into the pro-*S* position.¹⁴⁷ Hence, the relative orientation of substrate alcohol and NAD⁺ in the active site can be depicted (Figure 28).

As an enzyme that carries out an oxidation of a cyclohexadienol substrate to yield an aromatic product, EntA is analogous to prephenate dehydrogenase, an enzyme in the tyrosine biosynthetic pathway that cat-

SCHEME XIV



alyzes the oxidation/aromatization of prephenate (54) with loss of CO₂ to yield (*p*-hydroxyphenyl)pyruvate (101) (Scheme XIV). Cleland et al. have investigated the mechanism of this enzyme using a dihydro analogue substrate as well as kinetic studies measuring double-isotope effects.¹⁴⁹ These studies suggested that the enzyme-catalyzed reaction with prephenate was concerted with decarboxylation occurring in the same transition state as hydride removal. However, when deoxydihydroprephenate (which is missing the side chain keto group and one double bond in the ring) was the substrate, decarboxylation of the ensuing vinylogous β -keto acid did not occur, indicating a switch in mechanisms from concerted to stepwise when aromatization could not occur. These results raise the question of whether EntA-catalyzed oxidation/aromatization of 2,3-diHDB proceeds via a concerted mechanism also, but in this case, there is no comparable mechanism involving alcohol oxidation and aromatization to catechol. A concerted mechanism must involve a switch to extraction of either the C₂ or C₃ hydrogen (as hydride) with ejection of a proton from the other. The current results however strongly support the notion that EntA functions as an alcohol dehydrogenase, with the dehydrogenation occurring separate from aromatization.

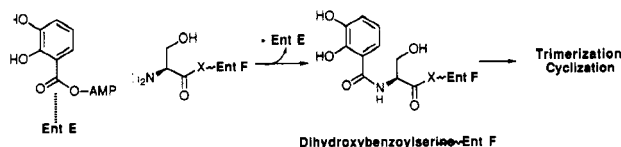
It may be that EntA evolved from an ancestral alcohol dehydrogenase to its present form such that the specificity of the predecessor remains to some extent. Dihydrodiol dehydrogenases converting *cis*-dihydrodiols to catechols have been described in bacterial transformations of aromatic compounds.^{150,151} However, a search of the protein data bank turned up no significant homologies except for a common NAD⁺ binding motif, even when compared with the sequence of *E. coli* shikimate dehydrogenase (the *aroE* gene product), an enzyme that catalyzes an oxidation with similar stereo- and regiochemical requirements as EntA.¹⁵²

D. Biosynthesis of Enterobactin from L-Serine and 2,3-Dihydroxybenzoate

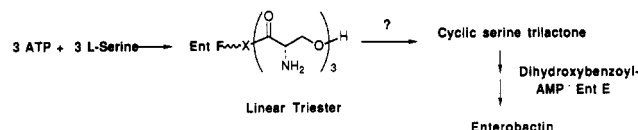
With many mechanistic details of the conversion of chorismate to 2,3-dihydroxybenzoate documented in the previous section, we now turn our attention to the rest of the pathway in which 2,3-dihydroxybenzoate and L-serine are converted to the macrocycle enterobactin. In these steps the mechanistic questions of concern include the details of formation of three amide and three ester bonds of the complete macrocycle and also the particular processes involving the cyclization and export of the siderophore to the environment. Scheme XV presents two possible routes for the stepwise as-

SCHEME XV

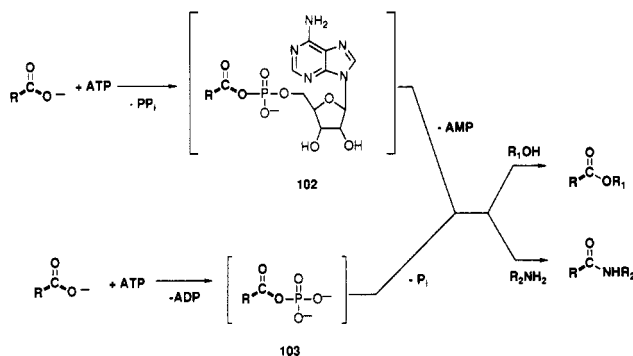
A. Initial Amide Bond Formation



B. Initial Ester Bond Formation



SCHEME XVI

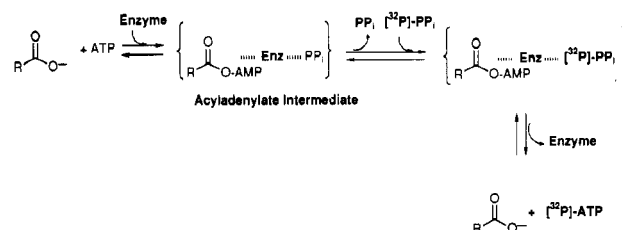


sembly of enterobactin in which amide bond formation either precedes or follows ester bond formation. Both of these mechanisms represent the growing polymer as remaining moored to one of the polypeptides involved in its synthesis, in this case EntF. The reasons for the choice of EntF will become apparent in the following discussion.

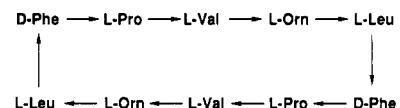
The formation of amide and ester bonds is an energy-requiring process, and with few exceptions, ATP provides the driving force for these reactions. The process by which these bonds are formed biochemically requires the activation of the carboxylic acid group by ATP to produce either an acyl adenylate 102 or an acyl phosphate 103 in which nucleophilic attack of the carboxylate on one of the phosphate group of ATP results in the formation of an acid-phosphoryl mixed anhydride (Scheme XVI). Ensuing nucleophilic attack by either an alcohol or amine on, for example, the acyl adenylate forms the requisite ester and amide bonds, respectively. Since the formation of the three amide and intramolecular lactone bonds in enterobactin is presented via acyl adenylate intermediates (i.e., six ATP molecules are cleaved to AMP and pyrophosphate products), we will concern ourselves exclusively with this avenue of carboxylate activation.

It is interesting that nature has chosen this highly activated intermediate to be used in the formation of acyl derivatives since aminoacyl adenylates are notoriously unstable in aqueous solution with half-lives of about 5–10 min at pH 7.2.¹⁶³ Apparently, these compounds remain bound to their generating enzymes where they are sequestered from solvent, rendering them at least kinetically stable. One of the tell-tale indications that an acyl adenylate participates as an intermediate in the mechanism is the observation of

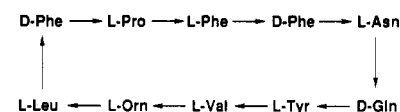
SCHEME XVII



SCHEME XVIII



104



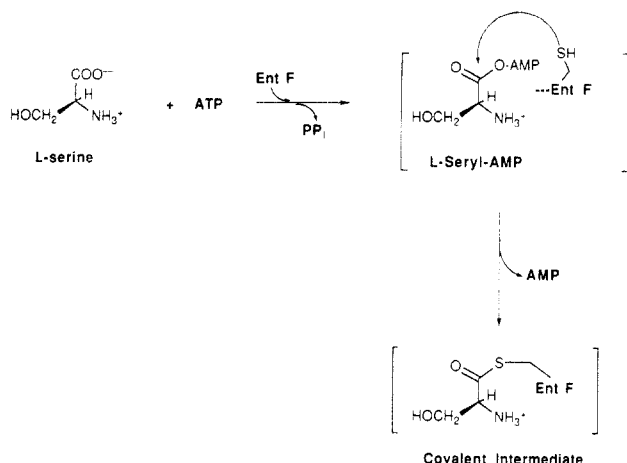
105

[³²P]pyrophosphate exchange into ATP, dependent upon carboxylate substrate, diagrammed in Scheme XVII.

Two of the best understood enzyme systems that catalyze amide bond formation through acyl adenylate intermediates are gramicidin synthase and tyrocidine synthase, which are involved in the biosynthesis of cyclic peptide antibiotics gramicidin S (104) and tyrocidine (105) (Scheme XVIII) from *Bacillus brevis*.^{154–159} Both gramicidin and tyrocidine synthases are comprised of two large polypeptides that provide the template for directed peptide synthesis. Following initial activation to the aminoacyl adenylate, a nucleophilic group on the enzyme displaces AMP to form a covalently bound amino acid. Although the group forming the covalent adduct has not been rigorously identified, it is believed to be a thiol since these covalent complexes are stable under acidic conditions; cysteine or covalently bound 4'-phosphopantetheine have been implicated.^{155–159} The covalent attachment of enzyme to the amino acid via the carboxylate serves not only to anchor the amino acid in place for the ensuing reaction but also to maintain an activated carboxylate group for subsequent amide (and ester bond) formation. It is proposed that chain elongation follows from the amino terminus by the attack of the free amino group of one anchored amino acid on the thioester group of another, forming a dipeptide that remains bound to the enzyme (via a thioester) at the carboxy terminus of the growing peptide.

An analogous system may well be involved in the enterobactin biosynthetic apparatus. As has been briefly alluded to in section III.A.3, there are four mutations called *entD*, *entE*, *entF*, and *entG* that were observed to affect the final assembly process. Early attempts to characterize the polypeptides associated with these genotypes showed that partially purified preparations of the EntE and EntF proteins catalyzed the ATP-[³²P]pyrophosphate exchange reaction dependent upon 2,3-dihydroxybenzoate and L-serine, respectively.^{160–163} Thus, carboxyl activation of both 2,3-dihydroxybenzoate and serine monomers proceeds through the representative acyl adenylates.

SCHEME XIX



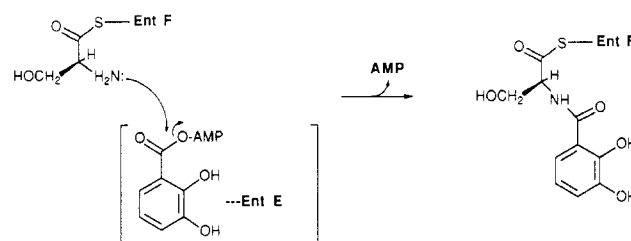
This has been confirmed for EntE with purified enzyme isolated from an overproducing strain of *E. coli*.^{164a} Incubation of EntE with [¹⁴C]salicylate (an analogue of 2,3-dihydroxybenzoate) followed by rapid gel filtration chromatography allowed isolation of EntE with associated radiolabeled substrate, and this was dependent upon ATP. Similar results were demonstrated with [¹⁴C]ATP but not with [γ -³²P]ATP, indicating that the species bound to EntE contained the carboxyl-containing substrate and the adenosine portion of ATP, but not the γ - (and β -) phosphates, compatible with an intermediate 2,3-dihydroxybenzoyl adenylate.

Incubation of an impure preparation of EntF with ATP and [¹⁴C]-L-serine followed by acid precipitation led to the isolation of EntF with [¹⁴C]-L-serine associated with the enzyme.¹⁶⁰ In analogy with gramicidin and tyrocidin synthases, it was proposed that serine becomes covalently attached to EntF via a thioester linkage (Scheme XIX). This contention was buttressed when it was demonstrated that hydroxylamine could liberate the radiolabeled amino acid from the enzyme.

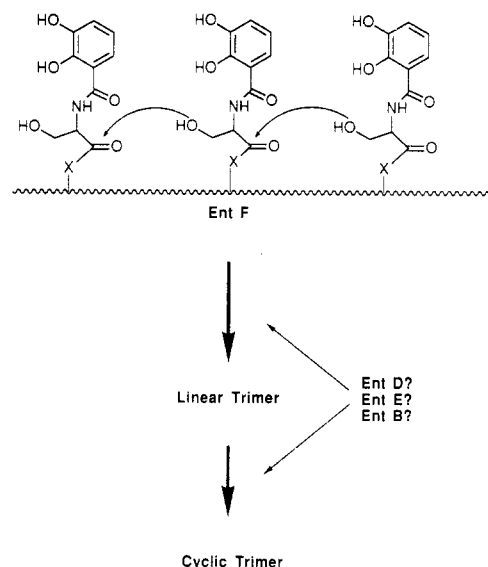
When similar experiments involving acid precipitation were performed with EntE and radiolabeled substrate, no label could be found associated with the enzyme.^{160,164a} Thus, it seems likely that after EntE reacts to form 2,3-dihydroxybenzoyl adenylate, this intermediate remains noncovalently bound to the enzyme and does not react to form a thioester. Furthermore, when impure fractions containing EntF, EntE, ATP, L-serine, and 2,3-dihydroxybenzoate (using radiolabeled serine or 2,3-dihydroxybenzoate) were incubated together, acid-stable intermediates were found associated with the protein fraction. In fact, intermediates corresponding to (2,3-dihydroxybenzoyl)serine, as well as the dimer and linear trimer of (dihydroxybenzoyl)serine were formed during this incubation.¹⁶⁰ Hence, it was proposed that the synthesis of enterobactin proceeds via reaction of the (2,3-dihydroxybenzoyl)serine complex of EntE with the thioester-linked complex of EntF to form an amide bond with the product, (2,3-dihydroxybenzoyl)serine, remaining covalently bound to EntF (Scheme XX).

Although the above studies were performed with enzymes for which the purity had not been assessed (except for EntE^{164a}), it is tempting to speculate further that trimerization of the (2,3-dihydroxybenzoyl)serine complex occurs on the protein template EntF, with three such molecules brought into proximity for step-

SCHEME XX



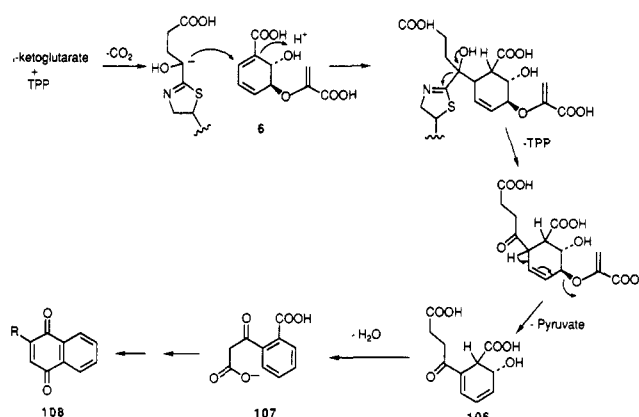
SCHEME XXI



wise ester bond formation to yield the linear form of enterobactin. Cyclization might then occur with the other components, of enterobactin synthase, namely EntD and EntG (Scheme XXI). Recently, the *entD* gene has been sequenced and shown to encode a polypeptide of molecular weight 23 600.^{111a,b} Sequence analysis suggested a transmembrane segment and expression analysis of the protein indicated that EntD is membrane-bound, probably in the *E. coli* inner membrane. Since enterobactin is such an efficient chelator of iron, it would seem preferential to complete its synthesis in a vectorial fashion, with the final product exported outside the cell at the same time as the final cyclization step. EntD may serve such a function.

The *entG* mutation, subsequent to its characterization and initial mapping,^{54,87} has since shown to be located within the open reading frame for the EntB polypeptide isochorismatase.⁵⁵ This leads to the exciting speculation that the EntB protein may actually be a bifunctional enzyme with two domains: the N-terminal domain necessary for isochorismatase activity, and the carboxy terminal domain required in the enterobactin synthase complex. In fact, antibodies raised against purified EntB have been shown to inhibit the enterobactin synthase reaction in vitro.^{164b} The possibility still exists that EntG may be a polypeptide distinct from EntB since DNA encoding only the 3'-end of the *entB* gene is able to complement the *entG* mutation.^{55,164b} Using site-directed mutagenesis to express a mutant EntB protein, which is missing a portion of the carboxy terminus, we have demonstrated that this protein still maintains full isochorismatase activity.¹⁶⁵ It should be a useful mutant to assess the postulated role of EntB as a component in macrocyclization.

SCHEME XXII



Further work to characterize more fully the eventual polymerization and cyclization reactions requires the purification of the other components of the enterobactin synthase complex, namely EntD and EntF. This is our current focus, and we are proceeding as outlined in section III.C.1 to overexpress these proteins for further mechanistic studies on the macrocycle assembly process.

IV. Other Transformations

While many other natural products are known to derive from chorismate or from isochorismate, we note here in detail only that isochorismate, formed via isochorismate synthase action as detailed in this chapter, is the key starting point for assembly of the naphthoquinone nucleus of vitamin K. The *menD* gene, the nucleotide sequence of which has recently been determined,¹⁶⁶ is known to process isochorismate and α -ketoglutarate to yield 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (106), with thiamin pyrophosphate as a cofactor.¹⁶⁷⁻¹⁶⁹ Further reaction catalyzed by the product of the *menC* gene converts this compound to *o*-succinylbenzoate (107) (Scheme XXII).

Acknowledgments. The work described here would not have been possible without the synthetic efforts of Professor Glenn Berchtold and his co-workers at the Massachusetts Institute of Technology. We are grateful to Professors Mark McIntosh, Charles Earhart, and Ian Young and their colleagues for providing some of the plasmids used in these studies and for helpful discussions and suggestions during these studies. We also acknowledge many helpful discussions with Dr. Stan Tabor and contributions of many former colleagues whose names appear in specific references. The financial support from the National Institute of Health (GM20011), a postdoctoral fellowship from NIH (GM12806-01, to F. R.), a postdoctoral fellowship from Suntory Institute for Bioorganic Research (Sunbor, Japan, to M.S.), and a Du Pont Graduate fellowship from the Massachusetts Institute of Technology (to J.L.) are gratefully acknowledged.

Registry No. 1, 617-12-9; enterobactin, 28384-96-5.

References

- Haslam, E. *The Shikimic Acid Pathway*; Halstead Press, Wiley: New York, 1974.
- Ganem, B. *Tetrahedron* **1978**, *34*, 3353.
- Weiss, U.; Edwards, J. M. *The Biosynthesis of Aromatic Compounds*; Wiley: New York, 1980.
- Conn, E. E., Ed. *The Shikimate Pathway*; Plenum: New York, London, 1986.
- Dewick, P. M. *Nat. Prod. Rep.* **1988**, *5*, 73.
- Welch, G. R.; Cole, K. W.; Gaertner, F. H. *Arch. Biochem. Biophys.* **1974**, *165*, 505.
- Hasan, N.; Nester, E. W. *J. Biol. Chem.* **1978**, *253*, 4993.
- White, P. J.; Millar, G.; Coggins, J. R. *Biochem. J.* **1988**, *251*, 313.
- Hill, R. K.; Newkome, G. R. *J. Am. Chem. Soc.* **1969**, *91*, 5893.
- Onderka, D. K.; Floss, H. G. *J. Am. Chem. Soc.* **1969**, *91*, 5894.
- Floss, H. G.; Onderka, D. K.; Carroll, M. *J. Biol. Chem.* **1972**, *247*, 736.
- Bartlett, P. A.; Maitra, U.; Chouinard, P. M. *J. Am. Chem. Soc.* **1986**, *108*, 8068.
- Bartlett, P. A.; McLaren, K. L.; Alberg, D. G.; Fassler, A.; Nyfeler, R.; Lauhon, C. T.; Grissom, C. B. In *Prospects for Amino Acid Biosynthesis Inhibitors in Crop Protection and Pharmaceutical Chemistry*; Copping, L. G., Dalziel, J., Dodge, A. D., Eds.; The British Crop Protection Council: Farnham, U.K., 1989.
- Guilford, W. J.; Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 5013.
- Gajewski, J. J.; Jurayj, J.; Kimbrough, D. R.; Gande, M. E.; Ganem, B.; Carpenter, B. K. *J. Am. Chem. Soc.* **1987**, *109*, 1170.
- Sogo, S. G.; Widlanski, T. S.; Hoare, J. H.; Grimshaw, C. E.; Berchtold, G. A.; Knowles, J. R. *J. Am. Chem. Soc.* **1984**, *106*, 2701.
- Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 5008.
- Andrews, P. R.; Smith, G. D.; Young, I. G. *Biochemistry* **1973**, *12*, 3492.
- Hudson, G. S.; Wong, V.; Davidson, B. E. *Biochemistry* **1984**, *23*, 6240.
- Addadi, L.; Jaffe, E. K.; Knowles, J. R. *Biochemistry* **1983**, *22*, 4494.
- Pawlak, J. L.; Padykula, R. E.; Kronis, J. D.; Aleksejczyk, R. A.; Berchtold, G. A. *J. Am. Chem. Soc.* **1989**, *111*, 3374.
- Jensen, R.; Fischer, R. *Methods Enzymol.* **1987**, *142*, 472.
- Gray, J. V.; Golinelli-Pimpaneau, B.; Knowles, J. R. *Biochemistry* **1990**, *29*, 376.
- Bartlett, P. A.; Johnson, C. R. *J. Am. Chem. Soc.* **1985**, *107*, 7792.
- Clarke, T.; Stewart, J. D.; Ganem, B. *Tetrahedron Lett.* **1987**, *28*, 6253.
- Bartlett, P. A.; Nakagawa, Y.; Johnson, C. R.; Reich, S. H.; Luis, A. *J. Org. Chem.* **1988**, *53*, 3195.
- Jackson, D. Y.; Jacob, J. W.; Sugawara, R.; Reich, S. H.; Bartlett, P. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, *110*, 4841.
- Hilvert, D.; Nared, K. D. *J. Am. Chem. Soc.* **1988**, *110*, 5593.
- Zalkin, H.; Kling, D. *Biochemistry* **1968**, *7*, 3566.
- Paluh, J. L.; Zalkin, H.; Betsch, D.; Weith, H. L. *J. Biol. Chem.* **1985**, *260*, 1889.
- Tso, J. Y.; Hermodson, M. A.; Zalkin, H. *J. Biol. Chem.* **1980**, *255*, 1451.
- Kawamura, M.; Keim, P. S.; Goto, Y.; Zalkin, H.; Heinrichson, R. L. *J. Biol. Chem.* **1978**, *253*, 4659.
- Crawford, I. P.; Eberly, L. *Mol. Biol. Evol.* **1986**, *3*, 436.
- Sato, S.; Nakada, Y.; Kanaya, S.; Tanaka, T. *Biochim. Biophys. Acta* **1988**, *950*, 303.
- Nichols, B. P.; Miozzari, G. F.; van Cleemput, M.; Bennett, G. N.; Yanofsky, C. *J. Mol. Biol.* **1980**, *142*, 503.
- Yanofsky, C.; Platt, T.; Crawford, I. P.; Nichols, B. P.; Christie, G. E.; Horowitz, H.; van Cleemput, M.; Wu, A. M. *Nucleic Acids Res.* **1981**, *9*, 6647.
- Bae, Y. M.; Holmgren, E.; Crawford, I. P. *J. Bacteriol.* **1989**, *171*, 3471.
- Zalkin, H.; Hwang, L. H. *J. Biol. Chem.* **1971**, *246*, 6899.
- Zalkin, H. *Methods Enzymol.* **1985**, *113*, 287.
- Polcastro, P. P.; Au, K. G.; Walsh, C. T.; Berchtold, G. A. *J. Am. Chem. Soc.* **1984**, *106*, 2443.
- Teng, C.-Y.; Ganem, B. *J. Am. Chem. Soc.* **1984**, *106*, 2463.
- Walsh, C. T.; Erion, M. D.; Waits, A. E.; Delany, J. J., III; Berchtold, G. A. *Biochemistry* **1987**, *26*, 4734.
- Kaplan, J. B.; Nichols, B. P. *J. Mol. Biol.* **1983**, *168*, 451.
- Goncharoff, P.; Nichols, B. P. *J. Bacteriol.* **1984**, *159*, 57.
- Nichols, B. P.; Seibold, A. M.; Doktor, S. Z. *J. Biol. Chem.* **1989**, *264*, 8597.
- Teng, C.-Y.; Ganem, B.; Doktor, S. Z.; Nichols, B. P.; Bhatmagar, R. K.; Vining, L. C. *J. Am. Chem. Soc.* **1985**, *107*, 5008.
- Francis, M. M.; Westlake, W. S. *Can. J. Microbiol.* **1979**, *25*, 1408.
- Shapiro, S.; Vining, L. C. *Can. J. Microbiol.* **1983**, *29*, 1706.
- Young, I. G.; Batterham, T. J.; Gibson, F. *Biochim. Biophys. Acta* **1968**, *165*, 567.
- Young, I. G.; Batterham, T. J.; Gibson, F. *Biochim. Biophys. Acta* **1969**, *177*, 389.
- Young, I. G.; Gibson, F. *Biochim. Biophys. Acta* **1969**, *177*, 401.

- (52) Laird, A. J.; Ribbons, D. W.; Woodrow, G. C.; Young, I. G. *Gene* 1980, 11, 347.
- (53) Laird, A. J.; Young, I. G. *Gene* 1980, 11, 359.
- (54) Pickett, C. L.; Hayes, L.; Earhart, C. F. *FEMS Microbiol. Lett.* 1984, 24, 77.
- (55) Nahlik, M. S.; Fleming, T. P.; McIntosh, M. A. *J. Bacteriol.* 1987, 169, 4163.
- (56) Liu, J.; Duncan, K.; Walsh, C. T. *J. Bacteriol.* 1989, 171, 791.
- (57) Elkins, M. F.; Earhart, C. F. *FEMS Microbiol. Lett.* 1988, 56, 35.
- (58) Ozenberger, B. A.; Brickman, T. J.; McIntosh, M. A. *J. Bacteriol.* 1989, 171, 775.
- (59) Liu, J.; Quinn, N.; Berchtold, G. A.; Walsh, C. T. *Biochemistry* 1990, 29, 1417.
- (60) Heyde, E.; Morrison, J. F. *Biochemistry* 1978, 17, 1573.
- (61) Young, I. G.; Jackman, L. M.; Gibson, F. *Biochim. Biophys. Acta* 1969, 177, 381.
- (62) Briggs, G. E.; Haldane, J. B. S. *Biochem. J.* 1925, 19, 338.
- (63) DeMarinis, R. M.; Filer, C. N.; Waraszkiewicz, S. M.; Berchtold, G. A. *J. Am. Chem. Soc.* 1974, 96, 1193.
- (64) Fukuyama, T.; Kishi, Y. *J. Am. Chem. Soc.* 1976, 98, 6723.
- (65) Law, W. C.; Kim, S.; Rando, R. R. *J. Am. Chem. Soc.* 1989, 111, 793.
- (66) Neurath, H. *Science* 1984, 224, 350.
- (67) Cotton, F. A.; Wilkinson, G. *Advanced Inorganic Chemistry*; Wiley: New York, 1980; p 281.
- (68) Deslongchamps, P. *Stereochemical Effects in Organic Chemistry*; Pergamon: Oxford, 1983.
- (69) Lawrence, J.; Cox, G. B.; Gibson, F. *J. Bacteriol.* 1974, 118, 41.
- (70) Neilands, J. B. In *Inorganic Biochemistry*; Eichorn, G. I., Ed.; Elsevier: New York, 1975; Vols. 1 and 2, p 167.
- (71) Neilands, J. B. *Annu. Rev. Biochem.* 1981, 50, 715.
- (72) Neilands, J. B.; Ratledge, C. In *CRC Handbook of Microbiology*, 2nd ed.; Laskin, A. I., Lechevalier, H. A., Eds.; CRC Press: Boca Raton, FL, 1982; Vol. IV, p 565.
- (73) van der Helm, D.; Jalal, M. A. F.; Hossain, M. B. In *Iron Transport in Microbes, Plants, and Animals*; Winkelmann, G., van der Helm, D., Neilands, J. B., Eds.; VCH: Verlagsgesellschaft, Weinheim, 1987; p 135.
- (74) Smith, M. J. *Tetrahedron Lett.* 1989, 30, 313.
- (75) Harris, W. R.; Carrano, C. J.; Cooper, S. R.; Sofen, S. R.; Avdeef, A. E.; McArdle, J. V.; Raymond, K. N. *J. Am. Chem. Soc.* 1979, 101, 6097.
- (76) Lee, C.-W.; Ecker, D. J.; Raymond, K. N. *J. Am. Chem. Soc.* 1985, 107, 6920.
- (77) Cass, M. E.; Garrett, T. M.; Raymond, K. N. *J. Am. Chem. Soc.* 1989, 111, 1677.
- (78) Neilands, J. B.; Erickson, T. J.; Rastetter, W. H. *J. Biol. Chem.* 1981, 256, 3831.
- (79) Bhattacharyya, S.; Corey, E. J. *Tetrahedron Lett.* 1977, 45, 3919.
- (80) Rastetter, W. H.; Erickson, T. J.; Venuti, M. C. *J. Org. Chem.* 1980, 45, 5012.
- (81) Shanzer, A.; Libman, J. *J. Chem. Soc., Chem. Commun.* 1983, 14, 846.
- (82) Earhart, C. F. In *Iron Transport in Microbes, Plants, and Animals*; Winkelmann, G., van der Helm, D., Neilands, J. B., Eds.; VCH: Verlagsgesellschaft, Weinheim, 1987; p 67.
- (83) Pollack, J. R.; Neilands, J. B. *Biochem. Biophys. Res. Commun.* 1970, 38, 989.
- (84) O'Brien, I. G.; Gibson, F. *Biochim. Biophys. Acta* 1970, 215, 393.
- (85) Young, I. G.; Langman, L.; Luke, R. K. J.; Gibson, F. *J. Bacteriol.* 1971, 106, 51.
- (86) Luke, R. K. J.; Gibson, F. *J. Bacteriol.* 1971, 107, 557.
- (87) Woodrow, G. C.; Young, I. G.; Gibson, F. *J. Bacteriol.* 1975, 124, 1.
- (88) McIntosh, M. A.; Earhart, C. F. *Biochem. Biophys. Res. Commun.* 1976, 70, 315.
- (89) Ichihara, S.; Mizushima, S. *J. Biochem.* 1978, 83, 137.
- (90) Lundrigan, M. D.; Kadner, R. J. *J. Biol. Chem.* 1986, 261, 10797.
- (91) Wookey, P.; Rosenberg, H. *J. Bacteriol.* 1978, 133, 661.
- (92) Pierce, J. R.; Pickett, C. L.; Earhart, C. F. *J. Bacteriol.* 1983, 155, 330.
- (93) (a) Pierce, J. R.; Earhart, C. F. *J. Bacteriol.* 1986, 166, 930. (b) Elkins, M. F.; Earhart, C. F. *J. Bacteriol.* 1989, 171, 5443.
- (94) (a) Ozenberger, B. A.; Nahlik, M. S.; McIntosh, M. A. *J. Bacteriol.* 1987, 169, 3638. (b) Chenault, S.; Elkins, M.; Earhart, C. F. Personal communication. (c) McIntosh, M. A. Personal communication. (d) Staudenmaier, H.; Van Hove, B.; Yaraghi, Z.; Braun, B. *J. Bacteriol.* 1989, 171, 2626.
- (95) Jalal, M. A. F.; van der Helm, D. *FEBS Lett.* 1989, 243, 366.
- (96) Kadner, R. J.; Bassford, P. J. In *Bacterial Transport*; Rosen, B. P., Ed.; Marcel Dekker: New York, 1978; p 413.
- (97) Hantke, K.; Zimmermann, L. *FEMS Microbiol. Lett.* 1981, 12, 31.
- (98) Langman, L.; Young, I. G.; Frost, G. E.; Rosenberg, H.; Gibson, F. *J. Bacteriol.* 1972, 112, 1142.
- (99) Heidinger, S.; Braun, V.; Pecoraro, V. L.; Raymond, K. N. *J. Bacteriol.* 1983, 153, 109.
- (100) Matzanke, B. F.; Ecker, D. J.; Yang, T.-S.; Huynh, B. H.; Müller, G.; Raymond, K. N. *J. Bacteriol.* 1986, 167, 674.
- (101) McIntosh, M. A. Personal communication.
- (102) Laird, A. J.; Ribbons, D. W.; Woodrow, G. C.; Young, I. G. *Gene* 1980, 11, 347.
- (103) Fleming, T. P.; Nahlik, M. S.; McIntosh, M. A. *J. Bacteriol.* 1983, 156, 1171.
- (104) Codere, P. E.; Earhart, C. F. *FEMS Microbiol. Lett.* 1984, 25, 111.
- (105) Flemming, T. P.; Nahlik, M. S.; Neilands, J. B.; McIntosh, M. A. *Gene* 1985, 34, 47.
- (106) Pettis, G. S.; McIntosh, M. A. *J. Bacteriol.* 1987, 169, 4154.
- (107) Pettis, G. S.; Brickman, T. J.; McIntosh, M. A. *J. Biol. Chem.* 1988, 263, 18857.
- (108) Nahlik, M. S.; Brickman, T. J.; Ozenberger, B. A.; McIntosh, M. A. *J. Bacteriol.* 1989, 171, 784.
- (109) Staab, J. F.; Elkins, M. F.; Earhart, C. F. *FEMS Microbiol. Lett.* 1989, 59, 15.
- (110) Tummuru, M. K. R.; Brickman, T. J.; McIntosh, M. A. *J. Biol. Chem.* 1989, 264, 20547.
- (111) (a) Armstrong, S. K.; Pettis, G. S.; Forrester, L. J.; McIntosh, M. A. *Mol. Microbiol.* 1989, 3, 757. (b) Codere, P. E.; Earhart, C. F. *J. Gen. Microbiol.* 1989, 135, 3043.
- (112) Ernst, J. F.; Bennett, R. L.; Rothfield, L. I. *J. Bacteriol.* 1978, 135, 928.
- (113) Hantke, K. *Mol. Gen. Genet.* 1984, 182, 288.
- (114) Schaffer, S.; Hantke, K.; Braun, V. *Mol. Gen. Genet.* 1985, 200, 110.
- (115) Bagg, A.; Neilands, J. B. *Biochemistry* 1987, 26, 5471.
- (116) DeLorenzo, V.; Wee, S.; Herrero, M.; Neilands, J. B. *J. Bacteriol.* 1987, 169, 2624.
- (117) De Lorenzo, V.; Herrero, M.; Giovannini, F.; Neilands, J. B. *Eur. J. Biochem.* 1988, 173, 537.
- (118) Wee, S.; Neilands, J. B.; Bittner, M. L.; Hemming, B. C.; Haymore, B. L.; Seetharam, R. *Biol. Met.* 1988, 1, 62.
- (119) (a) DeLorenzo, V.; Giovannini, F.; Herrero, M.; Neilands, J. B. *J. Mol. Biol.* 1988, 203, 875. (b) Neilands, J. B.; Nakamura, K. In *Microbial Iron Chelates*; Winkelmann, G., Ed.; CRC Press: Boca Raton, FL, in press.
- (120) Bagg, A.; Neilands, J. B. *Microbiol. Rev.* 1987, 51, 509.
- (121) Calderwood, S. B.; Mekalanos, J. J. *J. Bacteriol.* 1987, 169, 4759.
- (122) Calderwood, S. B.; Mekalanos, J. J. *J. Bacteriol.* 1988, 170, 1015.
- (123) Wosham, P. L.; Knonisky, J. *J. Bacteriol.* 1985, 161, 428.
- (124) (a) Niederhoffer, E. C.; Naranjo, C. M.; Fee, J. A. In *Metal Ion Homeostasis: Molecular Biology and Chemistry*; Hammer, D. H., Winge, D. R., Eds.; Alan R. Liss: New York, 1989; p 149. (b) Niederhoffer, E. C.; Naranjo, C. M.; Bradly, K. L.; Fee, J. A. *J. Bacteriol.* 1990, 1930.
- (125) Tabor, S.; Richardson, C. C. *Proc. Natl. Acad. Sci. U.S.A.* 1985, 82, 1074.
- (126) Rusnak, F.; Liu, J.; Quinn, N.; Berchtold, G. A.; Walsh, C. T. *Biochemistry* 1990, 29, 1425.
- (127) Busch, F. R.; Berchtold, G. A. *J. Am. Chem. Soc.* 1983, 105, 3346.
- (128) Addadi, L.; Jaffe, E. K.; Knowles, J. R. *Biochemistry* 1983, 22, 4494.
- (129) Berchtold, G. A. Personal communication.
- (130) Andrews, P. R.; Smith, G. D.; Young, I. G. *Biochemistry* 1973, 12, 3492.
- (131) Young, I. G.; Gibson, F. *Biochim. Biophys. Acta* 1969, 177, 182.
- (132) Rusnak, F.; Walsh, C. T. Unpublished results.
- (133) Marletta, M. A.; Cheung, Y.-F.; Walsh, C. T. *Biochemistry* 1982, 21, 2637.
- (134) Bondinell, W. E.; Vnek, J.; Knowles, P. F.; Sphrecher, M.; Sprinson, D. B. *J. Biol. Chem.* 1971, 246, 6191.
- (135) Cassidy, P. J.; Kahan, F. M. *Biochemistry* 1973, 12, 1364.
- (136) Zemell, R. I.; Anwar, R. A. *J. Biol. Chem.* 1975, 250, 3185.
- (137) Zemell, R. I.; Anwar, R. A. *J. Biol. Chem.* 1975, 250, 4959.
- (138) Warner, H. R.; Lands, W. E. M. *J. Biol. Chem.* 1961, 236, 2404.
- (139) Ellingson, J. S.; Lands, W. E. M. *Lipids* 1968, 3, 111.
- (140) Gunawan, J.; Debusch, H. *Hoppe-Seyler's Z. Physiol. Chem.* 1981, 362, 445.
- (141) Freeman, N. M.; Carey, E. M.; Carruthers, A. *Neurochem. Int.* 1984, 6, 273.
- (142) Anderson, K. S.; Sikorski, J. A.; Benesi, A. J.; Johnson, K. A. *J. Am. Chem. Soc.* 1988, 110, 6577.
- (143) Grimshaw, C. E.; Sogo, S. G.; Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* 1984, 106, 2699-2700.
- (144) Lee, J. J.; Asano, Y.; Shieh, T.-L.; Spreafico, F.; Lee, K.; Floss, H. G. *J. Am. Chem. Soc.* 1984, 106, 3367.
- (145) Asano, Y.; Lee, J. J.; Shieh, T. L.; Spreafico, F.; Kowal, C.; Floss, H. G. *J. Am. Chem. Soc.* 1985, 107, 4314.
- (146) Alber, D. G.; Bartlett, P. A. *J. Am. Chem. Soc.* 1989, 111, 2337.

- (147) Sakaitani, M.; Rusnak, F.; Quinn, B.; Frigo, T. B.; Tu, C.; Berchtold, G. A.; Walsh, C. T. *Biochemistry* 1990, in press.
- (148) Young, I. G.; Gibson, F. *Biochim. Biophys. Acta* 1969, 177, 348.
- (149) Hermes, J. D.; Tipton, P. A.; Fisher, M. A.; O'Leary, M. H.; Morrison, J. F.; Cleland, W. W. *Biochemistry* 1984, 23, 6263.
- (150) Axcell, B. C.; Geary, P. J. *Biochem. J.* 1973, 136, 927.
- (151) Rogers, J. E.; Gibson, D. T. *J. Bacteriol.* 1971, 130, 1117.
- (152) Anton, I. A.; Coggins, J. R. *Biochem. J.* 1988, 249, 319.
- (153) Moldave, K.; Castelfranco, P.; Meister, A. *J. Biol. Chem.* 1959, 234, 841.
- (154) Kleinkauf, H.; van Döhren, H. *Annu. Rev. Microbiol.* 1987, 41, 259.
- (155) Gevers, W.; Kleinkauf, H.; Lipmann, F. *Proc. Natl. Acad. Sci. U.S.A.* 1968, 60, 269.
- (156) Gevers, W.; Kleinkauf, H.; Lipmann, F. *Proc. Natl. Acad. Sci. U.S.A.* 1968, 63, 1335.
- (157) Roskoski, R.; Kleinkauf, H.; Gevers, W.; Lipmann, F. *Biochemistry* 1970, 9, 4839.
- (158) Roskoski, R.; Gevers, W.; Kleinkauf, H.; Lipmann, F. *Biochemistry* 1970, 9, 4846.
- (159) Lee, S. G.; Lipmann, F. *Methods Enzymol.* 1975, 43, 585.
- (160) Bryce, G. F.; Brot, N. *Biochemistry* 1972, 11, 1708.
- (161) Greenwood, K. T.; Luke, R. K. *J. Biochim. Biophys. Acta* 1976, 454, 285.
- (162) Woodrow, G. C.; Young, I. G.; Gibson, F. *Biochim. Biophys. Acta* 1979, 582, 145.
- (163) Greenwood, K. T.; Luke, R. K. *J. Biochim. Biophys. Acta* 1980, 614, 185.
- (164) (a) Rusnak, F.; Faraci, W. S.; Walsh, C. T. *Biochemistry* 1989, 28, 6827. (b) Staab, J.; Earhart, C. F. Personal communication.
- (165) Rusnak, F.; Walsh, C. T. Unpublished results.
- (166) Popp, J. L. *J. Bacteriol.* 1989, 171, 4349.
- (167) Weische, A.; Leistner, E. *Tetrahedron Lett.* 1985, 26, 1487.
- (168) Weische, A.; Johanni, M.; Leistner, E. *Arch. Biochem. Biophys.* 1987, 256, 212.
- (169) Weische, A.; Garvert, W.; Leistner, E. *Arch. Biochem. Biophys.* 1987, 256, 223.