Replacement by Site-directed Mutagenesis Indicates a Role for Histidine 170 in the Glutamine Amide Transfer Function of Anthranilate Synthase*

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Anthranilate synthase is a glutamine amidotransferase that catalyzes the first reaction in tryptophan biosynthesis. Conserved amino acid residues likely to be essential for glutamine-dependent activity were identified by alignment of the glutamine amidotransferase domains in four different enzymes: anthranilate synthase component II (AS II), p-aminobenzoate synthase component II, GMP synthetase, and carbamoyl-P synthetase. Conserved amino acids were mainly localized in three clusters. A single conserved histidine, AS II His-170, was replaced by tyrosine using site-directed mutagenesis. Glutamine-dependent enzyme activity was undetectable in the Tyr-170 mutant, whereas the NH₃-dependent activity was unchanged. Affinity labeling of AS II active site Cys-84 by 6-diazo-5-oxonorleucine was used to distinguish whether His-170 has a role in formation or in breakdown of the covalent glutamyl-Cys-84 intermediate. The data favor the interpretation that His-170 functions as a general base to promote glutaminyllation of Cys-84. Reversion analysis was consistent with a proposed role of His-170 in catalysis as opposed to a structural function. These experiments demonstrate the application of combining sequence analyses to identify conserved, possibly functional amino acids, site-directed mutagenesis to replace candidate amino acids, and protein chemistry for analysis of mutationally altered proteins, a regimen that can provide new insights into enzyme function.

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*This work was supported by United States Public Health Service Grant GM24658. This publication is Journal Paper 40359 from the Purdue University Agricultural Experiment Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1704 solely to indicate this fact.

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The abbreviations used are: AS I, anthranilate synthase component I; AS II, anthranilate synthase component II; PABS II, p-aminobenzoate synthase component II; DON, 6-diazo-5-oxo-1-norleucine.

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glutamine amide transfer function by ancestral NH₃-dependent enzymes (3–8). Techniques of enzymology (3, 9–12), protein chemistry (3, 15–16), and, more recently, site-directed mutagenesis (7) have been utilized to study the structure and function of anthranilate synthase. This laboratory has reported evidence for a mechanism of glutamine amide transfer that involves covalent glutaminyl and glutamyl adducts with an AS II active site cysteine (1, 3, 13). The active site cysteine was identified in the AS II subunit from Pseudomonas putida (14) and Serratia marcescens (15). Specific replacement of active site cysteine 84 by glycine in S. marcescens AS II confirmed the role of this residue in glutamine amide transfer (7). Using techniques of chemical modification and protein chemistry we could not identify other residues that functioned in the glutamine amide transfer mechanism.

In this report we present the initial results of an alternative approach to identify residues that are essential for glutamine amide transfer function. Amino acids that are conserved in homologous glutamine amide transfer domains of four different glutamine amidotransferases were identified and are considered likely to have essential structural or catalytic roles. Analysis of a proposed mechanism for glutamine amide transfer suggests a requirement for general base-general acid catalysis. We, therefore, examined the effects on catalysis of replacing the only conserved histidine residue in S. marcescens AS II. Site-specific replacement of AS II His-170 by Tyr inactivates glutamine amide transfer function without affecting NH₃-dependent synthesis of anthranilate. Reversion analysis is consistent with a function of His-170 in catalysis as opposed to a structural role.

Experimental Procedures

Strains, Plasmids, and Phage—Escherichia coli strain JMB9, relevant genotype ΔtrpEGD was used as a recipient for plasmids carrying S. marcescens trp genes (7). The growth rates of strain JMB9 transformants were determined as described (7). Plasmid pP17 is a S. marcescens trpE trpG pBR322 recombinant (7) that utilizes a plasmid promoter for trp gene transcription. The AS I and AS II subunits are encoded by trpE and trpG, respectively. Plasmid pP20 is a pP17 derivative that contains the strong S. marcescens trp operon promoter (7), pP19 (trpE'G'ID') and pP21 (trpE'G'ID') are isogenic with pP17 and pP20, respectively, but have an AS II Cys-84 to Gly replacement (7). M13 phage for mutagenesis and DNA sequencing have been described (7).

Oligonucleotide-directed Mutagenesis—A 17-mer d(TTCGGGB- TAGATGCGA) was synthesized, purified, and annealed to a gapped heteroduplex (7) constructed from M13mp11w and M13mp11trpG- PouH630 exactly as described (7). Single lane dideoxy sequencing to screen for the trpG mutant and gene reconstruction in pBR322 were by standard methods (7). Plasmid pNA1 (trpE'G'D') is a pNA3 derivative carrying the S. marcescens trp operon promoter. Mutations were verified by DNA sequencing (17).
**Role of His-170 in Anthranilate Synthase**

**Enzyme Purification**—Wild type *S. marcescens* anthranilate synthase was purified from *E. coli* strain JMB9 (ΔtrpEGD)/pJP20 (trpE*G*D') (7). Anthranilate synthase having AS Gly-84 or Tyr-170 replacements was purified from the same bacterial strain bearing plasmid pJP19 (trpE+G+D+) or pNA4 (trpE+GZD'), respectively. Glutamine- and NH₃-dependent anthranilate synthase was assayed at 37 °C (10). Glutamine synthetase was purified from E. coli strain JMBS (ΔtrpEGD)/pJP20 wild type, 23 pg of Gly-84 or 6.9 pg of Tyr-170 anthranilate synthase reaction mixture containing 50 mM triethanolamine HC1 (pH 7.4), 10 mM glutamine, 0.1 mM choline chloride, 10 mM EDTA, and 1.8 µg of wild type, 23 µg of Gly-84 or 6.9 µg of Tyr-170 anthranilate synthase (10). Glutamate was determined by the glutamate dehydrogenase assay (18). A unit of activity corresponds to 1 nmol of product formation/min. Specific activity is expressed as units/mg. Protein was determined by the method of Lowry et al. (19).

**Affinity Labeling**—Anthranilate synthase (3.0 µM) was incubated at 23 °C with 3.3 µM [35S]DON (2400 cpm/nmol) (20), 100 µM choline chloride, and 50 mM RPFO (pH 7.4) in a volume of 50 µl (10). For mutant anthranilate synthase the DON concentration was increased to 33 µM. The reaction was terminated by addition of 2.5 nmol of unlabeled DON and 3.0 µmol of glutamine, and the alkylated enzyme was isolated by centrifugal gel filtration (21) using Sephadex G-50 (Fine).

**Selection of Revertants**—Strain JMB9 (ΔtrpEGD) bearing plasmids pJP19 (trpE*GIDD*) or pNA3 (trpE*G2D2*) cannot synthesize tryptophan and grow normally in M9 media (22), with 50 µg/ml ampicillin, containing 1 mM NH₄Cl as a nitrogen source because of defective anthranilate synthase glutamine amide transfer. Strain JMB9 bearing plasmid pJP17 (trpE*G*D') which encodes wild type anthranilate synthase yields colonies or grows to stationary phase in liquid culture within 24 h in low NH₃ media containing ampicillin since NH₃ is assimilated into glutamine which can be utilized by the wild type anthranilate synthase for tryptophan synthesis. Spontaneous revertants of strain JMB9 bearing plasmid pJP19 or pNA3 were selected in liquid media from a series of 10 separate cultures. Revertants were obtained after 3-5 days at 37 °C, and only one revertant was saved from each of the cultures. Spontaneous revertants of JMB9/pJP19 were also isolated directly on solid media after incubation for 3-5 days. Plasmid DNA was isolated (22) from revertants grown in LB (23) plus ampicillin and used to retransform strain JMB9. Transformants were screened on low NH₃ plates containing ampicillin, and representative clones were checked by enzyme assay, measurement of growth rate, and DNA sequencing. In some cases a second cycle of plasmid isolation and retransformation was performed to resolve mixtures as determined by DNA sequencing.

**RESULTS**

**Glutamine Amide Transfer Domains**—Previous attempts to detect essential AS II amino acids, other than Cys-84 (15), were unsuccessful using techniques of protein chemistry. Side reactions of group-specific reagents with Cys-84 were difficult to exclude. The present objective was, therefore, to identify conserved amino acids in all four sequences. By these criteria 27% of the 193 AS II amino acids have been conserved in all four sequences. By these criteria 11. Of the 19 identical amino acids, 13 are in three clusters at 198 of GMP synthetase, AS II, and PABS II in Fig. 1. The alignment shown in Fig. 1 has 19 identities and 34 positions with conserved residues in all four sequences. By these criteria 27% of the 193 AS II amino acids have been conserved in the trpG-related glutamine amide transfer domain in carA-encoded carbamoyl-P synthetase, GMP synthetase, and PABS II. Of the 19 identical amino acids, 13 are in three clusters at positions 57-64, 85-102, and 187-192. The single conserved cysteine at position 91, which corresponds to Cys-84 in *S. marcescens* AS II, was previously shown by affinity labeling (10, 15) and site-directed mutagenesis (7) to function in glutamine amide transfer. Our proposal for the mechanism of glutamine amide transfer (below) predicts a requirement for an amino acid side chain that can function in general acid-base catalysis to donate and abstract protons. The best candidates for such a group are the conserved histidine at position 190 and the conserved glutamate at position 192. Accordingly, we have replaced the histidine at position 190, histidine 170 in *S. marcescens* AS II.

**Mutant Isolation**—A CAC (His) to TAC (Tyr) change at codon 170 in *S. marcescens* trpG was constructed by incorporation of a synthetic oligonucleotide into a recombinant M13mp11-trpG heteroduplex. The nucleotide and amino acid sequence of the pertinent region of trpG together with the sequence of the synthetic 17-mer are shown in Fig. 2. The mutagenic 17-mer was annealed to the complementary region of the trpG non coding strand (Fig. 2), generating a C:A mismatch at nucleotide 508. Following primer extension, ligation, and transformation, trpG phage were screened by an "A lane" sequencing (Fig. 3). One mutant was identified among 48 phage screened. The Tyr-170 mutation was designated trpG2. The trpG2 mutation was transferred into plasmid pJP17 (trpE*G'D') to yield pNA3 (trpE*G2D2*) in which trp genes are transcribed from a pBR322 plasmid promotor.

The nucleotide sequence of trpG2 was checked between nucleotides 370-520. The only change from wild type was C to T at position 508. A portion of the sequencing gel that verifies the mutation is shown in Fig. 4.

**Enzyme Activity**—In order to examine the consequences of the trpG2 mutation, anthranilate synthase activity was determined in extracts of plasmid-bearing cells. Specific activities of 9.8 units/mg and 13.1 units/mg were obtained for the glutamine- and NH₃-dependent activities, respectively, for wild type anthranilate synthase from cells bearing plasmid pJP19 (trpE*GIDD*). Glutamine-dependent anthranilate synthase was undetectable from cells bearing plasmid pNA4 (trpE*G2D2*), whereas the NH₃-dependent activity was 18.7 units/mg. To confirm that the Tyr-170 replacement inactivated the glutamine amide transfer function of anthranilate synthase, the trpG2 mutant and wild type enzymes were purified to homogeneity. The NH₃-dependent activities were 2150 and 2280 units/mg for the wild type and trpG2 mutant, respectively. Glutamine-dependent anthranilate synthase was 3200 units/mg for the wild type and less than 0.01 unit/mg for the Tyr-170 mutant enzyme (trpE*G2D2*) encoded by pNA4. These data are formally similar to those obtained for the trpG1 mutant anthranilate synthase having an AS II Cys-84 to Gly replacement and, therefore, suggest that His-170 is required in addition to Cys-84 for glutamine amide transfer function.

**In Vivo Function**—Paluh et al. (7) reported that the NH₃-dependent activity of trpE*G1* (Gly-84)-encoded anthranilate synthase supported tryptophan synthesis and growth in minimal media containing 50 mM NH₄Cl but not in media containing 1 mM NH₄Cl. Quantitatively similar results were obtained for the trpE*G2 Tyr-170 mutant. The doubling time of strain JMB9 (ΔtrpEGD) bearing plasmid pNA4 (trpE*G2D2*) in minimal media containing 50 mM NH₄Cl was 1 h, in contrast to a 15-h doubling time in media containing 1 mM NH₄Cl. These results further support the conclusion (7) that the glutamine-dependent activity of anthranilate synthase provides selective advantage in media having limiting levels of NH₃.

**Role of His-170—Alkylation of AS II**—Cys-84 by the glutamine amide analog DON mimics formation of the catalytic glutaminyl-cysteine 84 covalent intermediate (3, 10). Affinity labeling of the trpE*G2 mutant enzyme was conducted to
Role of His-170 in Anthranilate Synthase

Fig. 1. Amino acid alignment of the glutamine amide transfer domains in carbamoyl-P synthetase (CPS), GMP synthetase (GMPS), AS II, and PABS II (PABS). The latter three enzymes were aligned by computer (29) and carbamoyl-P synthetase (24) was fitted by visual inspection. + indicates a match in all proteins, while . indicates conserved residues in all four proteins according to the following groups: (K,R); (S,T); (P,G); (Q,N,E,D); and (H,Y,W,F,I,L,V,M,C,A) with the latter large group corresponding to hydrophobic amino acids. The numbering scheme counts all positions, including gaps in the alignment of the four proteins. The numbers preceding position 1 denote the number in the protein chain of the first residue shown.

![Amino acid alignment](image)

Fig. 2. Nucleotide sequence of a pertinent region of trpG and of a synthetic oligonucleotide used for mutagenesis. The wild type sequence is shown on top. The nucleotide sequence of trpG is numbered from the start of translation. A synthetic 17-mer having a one-base mismatch (underlined) is shown below.

![Nucleotide sequence](image)

Fig. 3. Screening for a trpG mutant having a G to A base change in coding strand DNA. The photograph shows a portion of the dideoxy A reaction sequencing gel with 8 DNA samples. The arrows point to a mutant in lane 3 having the G to A base change.

![Screening for trpG mutant](image)

determine whether His-170 functions in a step prior to or after formation of the covalent glutaminyl enzyme intermediate. [14C]DON was rapidly incorporated into the wild type enzyme (Fig. 5) and concomitantly inactivated the glutamine-dependent activity (not shown). The stoichiometry for incorporation was approximately 0.7 eq of DON/AS I-AS II protomer. Mutant enzyme with the AS II Gly-84 replacement was not alkylated verifying the specificity of DON for AS I Cys-84. Using the same conditions as for the wild type enzyme, the Tyr-170 mutant also was not alkylated. However, a 10-fold increase in DON concentration permitted incorporation of DON at a rate approximately 2% that of the wild type (Fig. 5). The simplest interpretation of this result is that His-170 has an essential role in formation of the covalent...
Role of His-170 in Anthranilate Synthase

not detected for anthranilate synthase having the Gly-84 or Tyr-170 replacements, thus supporting the conclusion that the two mutant enzymes are defective in glutamine utilization.

Reversion Analysis—Evidence is required to distinguish whether an essential amino acid residue functions in catalysis or exerts a structural role. Analysis of revertants can potentially distinguish between these two cases. For the case of an amino acid that functions in catalysis, reversion of a missense mutation must restore that amino acid. For an amino acid that exerts a structural role, a different amino acid could be functional at the initial site or at a second site (27, 28). E. coli strain JMB9 (ΔtrpEGD) bearing plasmids trpE"GID" (AS II Gly-84) or trpE"GID" (AS II Tyr-170) does not grow on minimal agar media containing 1 mM NH₄Cl because mutant anthranilate synthase with defective glutamine amide transfer function cannot utilize glutamine for tryptophan synthesis. A low concentration of NH₄ can be used by glutamine synthetase but not by anthranilate synthase. We selected spontaneous revertants of strains JMB9 (ΔtrpEGD)/pJP19 (trpE"GID") Gly-84 anthranilate synthase and JMB9 (ΔtrpEGD)/pN4A (trpP"GID") Tyr-170 anthranilate synthase that utilized 1 mM NH₄Cl as a nitrogen source for tryptophan synthesis as described under "Experimental Procedures." For strain JMB9 (ΔtrpEGD)/pJP19 (trpE"GID"), five revertant colonies of varied sizes were obtained on solid media after 5 days. The reversion frequency was 1 in 4 × 10⁶. Twelve colonies of different sizes were picked for further analysis. Following plasmid isolation and retransformation all colonies appeared to grow at the same rate consistent with a single class of revertants. Furthermore, the growth of revertants initially isolated in liquid and on solid media was indistinguishable on agar plates. The growth rate in low NH₄Cl liquid culture of four revertant strains was measured and was identical to that of the parental JMB9 (ΔtrpEGD)/pJP17 (trpE"GID") strain. Likewise, glutamine-dependent anthranilate synthase activity was restored to the wild type level in extracts of the four strains examined. Finally, DNA sequence analysis indicated that 12/12 revertants had a GGC (Gly) to TGC (Cys) reversion at codon 84. In an identical manner, Tyr-170 revertants were obtained. Representative strains (10/10) grew in low NH₄ minimal media at the wild type rate, regained the wild type glutamine-dependent enzyme activity (10/10), and restored CAC (His) at codon 170 (10/10). These results support a role of Cys-84 and His-170 in catalysis.

DISCUSSION

All glutamine amidotransferases, including anthranilate synthase from nine prokaryotic and eukaryotic organisms (14, 29-32), utilize an active site cysteine in glutamine amide transfer function (2, 6, 33). S. marcescens AS II cysteine 84 was identified as the essential residue by affinity labeling (10, 15) and was confirmed by replacement with glycine using site-directed mutagenesis (7). Attempts to identify other residues that function in glutamine amide transfer using chemical modification by histidine and arginine reagents were unsuccessful because of the difficulty in eliminating the possibility that inactivation was due to modification of the highly reactive AS II Cys-84.² We have now utilized newly available techniques to identify residues likely to be important for glutamine amide transfer structure and function and have provided evidence for a role of AS II His-170 in catalysis.

The alignment of glutamine amide transfer domains in E. coli carbamoyl-P synthetase, GMP synthetase, AS II, and PABS component II, shown in Fig. 1, identifies three regions having highly conserved primary structure. It is likely that conserved residues in the homologous glutamine amide trans-
fer domains are critical for structure or catalysis. Indeed, *S. marcescens* AS II Cys-84 is conserved at position 91. In addition to the essential cysteine, the mechanism for glutamine amide transfer likely requires a group to abstract and donate protons (see below). AS II His-170, position 190 in Fig. 1, is a good candidate for such a residue, and the replacement of His-170 by tyrosine was shown to inactivate glutamine amide transfer without having any effect on NH₃-dependent activity.

Affinity labeling of Cys-84 by DON was used to provide evidence for the role of His-170 in glutamine amide transfer. The reactions shown in Fig. 7 summarize our view of glutamine amide transfer. The overall reaction (3) is divided into three steps, formation of the glutaminylation-cysteine enzyme adduct, amide transfer, and thioester hydrolysis. We visualize that in each step a general base (:B) or its conjugate general reactants leading to glutaminase activity. Since the rate of general base (:B) in step 1. According to the scheme, the affinity labeling by glutamine analogs mimics step 1 and dependent activity.

There are several possibilities to explain why affinity labeling of the Tyr-170 enzyme occurred at a slow rate, but glutaminase activity was not detected. DON may be more reactive to nucleophilic attack by Cys-84 than the carboxamide group of glutamine. Alternatively, step 1 may not be rate determining for glutamine hydrolysis.

The proposed role of His-170 in the glutaminylation of AS II Cys-84 (Fig. 7) is analogous to the function of His-159 in the acylation of Cys-25 by peptide substrates in papain (34). Furthermore, if the conserved AS II Glu-172, position 192 in Fig. 1, were to also participate in proton transfer, the triad Cys-84, His-170, and Glu-172 would be similar in function to the chymotrypsin charge-relay system composed of Ser-195, His-57, and Asp-102 (35). In this regard, it is of interest that chemical conversion of Ser-195 to alanine in chymotrypsin decreased the reactivity of His-57 to affinity labeling (36). In AS II replacement of His-170 decreased the reactivity of Cys-84 to affinity labeling (Fig. 5).

We recognize two main qualifications that bear upon the proposed role of AS II His-170 and the mechanism suggested in Fig. 7. First, the scheme in Fig. 7 describes the simplest possible case. Other amino acids could function together with AS II His-170 in abstracting and donating protons. Further mutagenesis experiments are required to clarify this possibility. Second, we need to evaluate the evidence that His-170 has a direct role in catalysis and not a structural role. As discussed below, reversal analysis provided preliminary evidence for a role of His-170 in catalysis.

In previous replacements of the active site cysteine in two glutamine amidotransferases, enzymatic, chemical, and physical properties of the mutant and wild type enzymes were compared and found to be indistinguishable (6, 7). This evidence supported the conclusions derived from affinity labeling that a cysteine residue is required for glutamine amide transfer. However, it is uncertain whether measurements of NH₃-dependent activity, allosteric inhibition, proteolytic inactivation, and circular dichroism are adequately sensitive to detect putative small local changes in conformation that could obstruct Cys-84 and disrupt glutamine amide transfer. Reversion analysis should provide an alternative sensitive distinction between residues that are required for catalysis or for structure. Amino acids that participate in catalysis should be irreplaceable whereas other amino acids should be able to correct a missense mutation by incorporation at the primary or secondary positions. Previous studies of *E. coli* tryptophan synthase provide examples for restored function of second site reversions in missense mutants (27, 28). In our experiments all trpE*G1D* and trpE*G2D* revertants were of a single class in which the original trpG mutation was corrected to restore the wild type codon. These results are consistent with a role of AS II His-170 in catalysis. Further evaluation of this approach is required to determine the relative frequency that missense mutations causing structural alterations can be corrected by the original amino acid compared to a structurally acceptable amino acid at the primary or secondary sites.

**Acknowledgments**—We thank Henry Weiner and Charles Yanofsky for reading the manuscript and offering suggestions. We thank R. L. Somerville for suggesting reversion analysis of trpG mutants.
Role of His-170 in Anthranilate Synthase

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