Mutagenic Analysis of an Invariant Aspartate Residue in Chorismate Synthase Supports Its Role as an Active Site Base†

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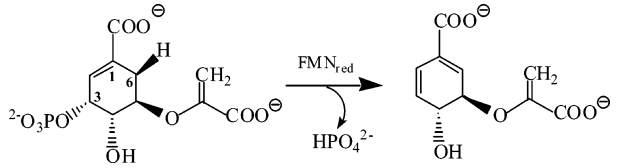
ABSTRACT: Chorismate synthase catalyzes the *anti*-1,4-elimination of the 3-phosphate and the C(6*proR*) hydrogen from 5-enolpyruvylshikimate 3-phosphate (EPSP) to generate chorismate, the final product of the common shikimate pathway and a precursor for the biosynthesis of aromatic compounds. The enzyme has an absolute requirement for reduced FMN, which is thought to facilitate cleavage of C−O bonds by transiently donating an electron to the substrate. The crystal structure of the enzyme revealed that EPSP is bound near the flavin isoalloxazine ring with several invariant amino acid residues in contact with the substrate and/or cofactor. Here, we report the results of a mutagenesis study in which an invariant aspartate residue at position 367 of the *Neurospora crassa* chorismate synthase was replaced with alanine and asparagine. Both single mutant proteins (Asp367Ala and Asp367Asn) were comparable to the wild-type enzyme with respect to substrate and cofactor binding, indicating that Asp367 is not required for binding of either the flavin or the substrate. In sharp contrast to these results, the activity of both single mutant proteins was found to be 620 and 310 times lower for the Asp367Ala and Asp367Asn mutant proteins, repectively. This finding provides strong evidence that the carboxylate group of Asp367 plays a major role during the catalytic reaction. On the basis of the structure of the enzyme, our data provide the first experimental evidence that the carboxylate group of aspartate 367 participates in the deprotonation of N(5) of the reduced flavin cofactor, which in turn abstracts the C(6*proR*) hydrogen yielding chorismate as the product.

Chorismate synthase is the seventh and last enzyme of the common shikimate pathway, which generates chorismate for further synthesis of aromatic compounds, such as the aromatic amino acids and many other essential products. Because the shikimate pathway is only present in prokaryotes, fungi, plants, and apicomplexa, its enzymes are interesting potential targets for the development of antibiotics (*1*). Chorismate synthase carries out the transformation of 5-enolpyruvylshikimate 3-phosphate (EPSP)[1] to chorismate, a reaction that involves the *anti*-1,4-elimination of the 3-phosphate and C(6*proR*) hydrogen, as shown in Scheme 1 (*2*, *3*).

Chorismate synthase activity also relies on the presence of reduced FMN, which is bound in the active site of the enzyme (*3*−*5*). The role of reduced FMN in this non-redox elimination reaction has been subjected to intensive studies over the past two decades (*6*, *7*), resulting in a reaction scheme in which the reduced FMN transiently donates an electron (or involves a charge transfer) to the substrate prompting cleavage of the C−O bond (*8*−*11*). Despite the accumulation of theoretical and experimental evidence of such a role of the reduced FMN in the chorismate synthase-catalyzed reaction, the mechanistic proposals could not be probed for a lack of structural information about the protein. Eventually, in 2003, the first crystal structure of the enzyme in complex with oxidized FMN and EPSP was reported and enabled us to initiate a structure-based mutagenesis program to test mechanistic proposals (*10*). The first targets were two invariant histidine residues which are located in the vicinity of the N(1)−C(2)═O locus of the flavin ring system and the 3-phosphate group of the substrate. Single mutant proteins in which the invariant histidine is replaced with alanine exhibit 10−20-fold lower activity, supporting their role as active site acids (*12*).

Scheme 1



A pivotal problem with regard to the transformation of EPSP to chorismate revolves around the acceptor that abstracts the C(6*proR*) hydrogen (formally as either a hydrogen atom or a proton). The crystal structure shows that only the N(5) atom of the isoalloxazine ring system is

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[1] Abbreviations: EPSP, 5-enolpyruvylshikimate 3-phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; *Nc*CS, *Neurospora crassa* chorismate synthase.

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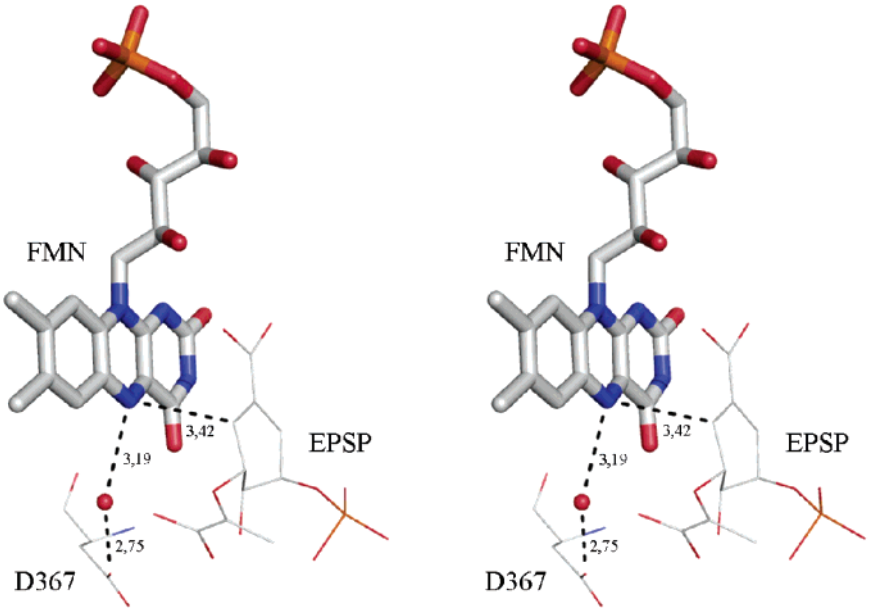


FIGURE 1: Asp367 residue in the chorismate synthase active site. Stereo representation of the interaction made by the Asp367 residue with the N(5) position of FMN via a conserved water molecule. The carbon atoms of Asp367, FMN, and EPSP are colored gray. The red sphere represents the conserved water position. Hydrogen bonds are shown as dashed lines.

in a position to abstract the hydrogen from the substrate (Figure 1). The relative orientation of the N(5) atom and the C(6*proR*) hydrogen also gives a rationale for the observed stereospecificity of the elimination reaction (*2*). The only amino acid that is close to the N(5) position of the flavin is Asp367 [in the reported crystal structure of the *Streptococcus pneumoniae* chorismate synthase, this position is designated as Asp339 (*10*)] engaging in a hydrogen bonding interaction with a bound water molecule which in turn is within hydrogen bonding distance of N(5) [note that N(5) bears a hydrogen in the reduced cofactor]. A plausible role for the carboxylate group of Asp367 involves the deprotonation of the N(5) hydrogen (from the reduced or semiquinone form) to prime the nitrogen for the abstraction of the substrate C(6*proR*) hydrogen (*10*). To analyze the putative role of this invariant aspartate residue, we have generated two single mutant proteins in which the aspartate has been replaced with either alanine or asparagine. In this report, we show that both replacements have caused a major decline in enzymatic activity consistent with the role of aspartate as an essential partner in acid−base catalysis in the active site of chorismate synthase.

MATERIALS AND METHODS

Reagents. All chemicals were of the highest available grade and were obtained from Sigma or Fluka (Buchs, Switzerland). DEAE Sephacel was from Amersham Biosciences, and cellulose phosphate (P11) was from Whatman (Kent, U.K.). DNA restriction and modification enzymes were obtained from Fermentas GmbH or New England Biolabs (Beverly, MA). The plasmid−DNA preparation was performed with the Nucleobond AX plasmid preparation kit (Macherey-Nagel GmbH & Co. KG). PCR primers were from VBC-Genomics (Vienna, Austria). EPSP was synthesized from shikimate 3-phosphate using recombinant *Escherichia coli* 5-enolpyruvylshikimate 3-phosphate synthase and purified by HPLC.

Molecular Techniques. Basic molecular manipulations were performed using standard techniques (*13*).

Site-Directed Mutagenesis. Amino acid replacements were performed using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The pET21a-*Nc*CS construct served as the template. The following oligonucleotides containing the appropriate codon exchange were used for the procedure (the changed codon is underlined): for D367A, forward primer (5′-GGGTAGACACGCCCCAGCGTGGTGC-3′) and reverse primer (5′-GCACCACGCTGGGGGCGTGTCTACCC-3′); and for D367N, forward primer (5′-CCAAGGGTAGACACAACCCCAGCGTGGTGC-3′) and reverse primer (5′-GCACCACGCTGGGGTTGTGTCTACCCTTGG-3′). All manipulations were performed following the manufacturer's instructions. The mutations were verified by DNA sequencing (MWG-Biotech AG).

Production and Purification of Neurospora crassa Chorismate Synthase (NcCS). The aspartate to alanine and aspartate to asparagine mutant proteins were produced and purified as described for the wild-type enzyme (*14*). The concentration of the purified mutant proteins was obtained with Ultracel YM-10 Centripreps (Amicon Bioseparations).

UV−Visible Absorbance Spectrophotometry. Absorbance spectra were recorded with a Specord 210 spectrophotometer equipped with a thermostated cell holder (Analytik Jena). All experiments were performed at 25 °C.

Activity Assay under Aerobic Conditions. A continuous enzyme assay was employed as described in ref *14*.

Stopped-Flow Spectrophotometry. Single- and multiple-turnover experiments were carried out using a Hi-Tech Scientific SF-61 stopped-flow spectrophotometer (Salisbury, U.K.) at 25 °C. The stopped-flow observation cell had a path length of 1.0 cm. Enzyme and substrate solutions were made anaerobic by exchanging the dissolved oxygen with argon via several cycles of evacuation and flushing. An anaerobic substrate solution was mixed with an anaerobic enzyme solution both containing $FMNH_2$ that was reduced using

Table 1: Dissociation Constants for FMN and EPSP

ligand	K_d (μM) wild-type *Nc*CS	Asp367Ala *Nc*CS	Asp367Asn *Nc*CS	method
FMN	41 ± 5^a	65 ± 8^a	58 ± 3^a	UV−visible difference spectroscopy
EPSP (with FMN)	17^b	17^b	16^b	UV−visible spectroscopy

[a] Average of three independent measurements. [b] Average of two independent measurements.

photoirradiation in the presence of potassium oxalate. Kinetic data were fitted using Hi-Tech Scientific KinetAsyst version 3.14.

Spectra of Reaction Intermediates. Absorbance spectra were recorded with a Hewlett-Packard photodiode array instrument (model HP8452) using a cuvette with a side arm. The enzyme solutions and the substrate in the side arm of the cuvette were made anaerobic by exchanging the dissolved oxygen with argon via several cycles of evacuation and flushing. The anaerobic enzyme solution containing $FMNH_2$ was reduced using photoirradiation in the presence of potassium oxalate before the sample was mixed with the substrate and the spectra were recorded.

RESULTS

Production and Purification of the Asp367Ala and Asp367Asn Mutant Chorismate Synthases. Production of the two Asp367Ala and Asp367Asn mutant proteins in *E. coli*, strain BL21(DE3) RP, resulted in protein levels comparable to those obtained with wild-type *N. crassa* chorismate synthase (data not shown). Both mutant proteins were purified according to the protocol developed for the purification of the wild-type protein and yielded ~3 mg of protein per gram of wet cell paste, again similar to the yields obtained previously with the wild-type protein (*14*). The mutant proteins were isolated as the apoprotein; i.e., the protein preparations were devoid of FMN, as is also the case for the wild-type protein.

Binding of FMN to the Mutant Proteins. Since the introduced mutation is in the direct vicinity of the N(5) atom of the flavin isoalloxazine ring system, it is important to ensure that the cofactor binds to the active site of the mutant proteins. To demonstrate the binding of the cofactor, both mutant proteins were titrated with FMN, and binding was monitored using difference UV−vis spectrophotometry (Figure 2). The spectral perturbations observed when FMN was added to either the Asp367Ala (not shown) or Asp367Asn (Figure 2) mutant protein were identical to those observed for the wild-type enzyme (*14*). In addition, the dissociation constants determined in these experiments showed only slightly weaker binding of the cofactor to the mutant proteins (inset of Figure 2 and Table 1).

Binding of EPSP to the Mutant Proteins. Binding of the substrate EPSP produces perturbations of the UV−vis absorbance spectrum of oxidized FMN which can be exploited in determining the dissociation constant of the substrate. As shown in Figure 3, the observed spectral changes when EPSP binds to the Asp367Asn mutant protein are identical to those observed with wild-type chorismate synthase (*14*). Moreover, binding exhibits the same dissociation constant as previously determined for the wild-type enzyme. The same spectral perturbations and dissociation constant were found with the Asp367Ala mutant protein (Table 1).

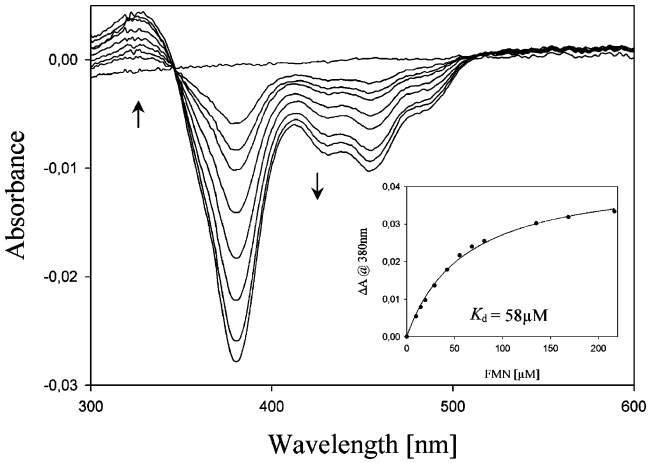


FIGURE 2: Binding of oxidized FMN to the Asp367Asn mutant protein. The plot shows titration of the Asp367Asn mutant protein (20 μM) with oxidized FMN in 50 mM MOPS buffer (pH 7.5). Arrows indicate the direction of the spectral changes occurring upon titration with FMN. Difference absorbance spectra at 0, 9.8, 14.6, 19.4, 29, 42.2, 55.3, 80.9, and 93.5 μM oxidized FMN are shown. The inset shows the spectral changes at 380 nm as a function of FMN concentration, revealing a dissociation constant (K_d) of 58 μM.

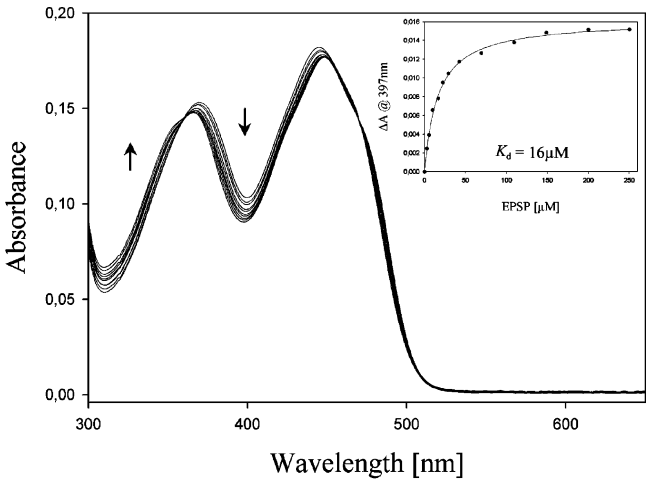


FIGURE 3: Binding of EPSP to the Asp367Asn mutant protein in the presence of oxidized FMN. Titration of the Asp367Asn mutant protein with EPSP in 50 mM MOPS buffer (pH 7.5). UV−visible absorbance spectra of the Asp367Asn mutant protein (20 μM) and FMN (18 μM) were recorded at various EPSP concentrations. The spectra that are shown were recorded at the following EPSP concentrations: 0, 2.8, 5.7, 9.9, 16.9, 22.3, 29.1, 42.4, 69.4, and 109.3 μM. Arrows indicate the direction of the absorbance changes. The inset shows the spectral changes at 397 nm as a function of EPSP concentration, revealing a K_d of 16 μM.

NADPH Oxidase Activity of the Mutant Proteins. N. crassa chorismate synthase belongs to the family of bifunctional

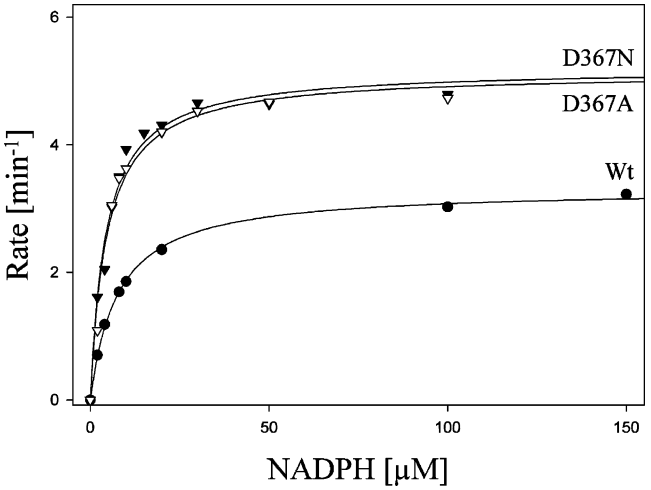


FIGURE 4: Oxidation of NADPH by wild-type (Wt) *Nc*CS and the two aspartate mutant proteins under aerobic conditions. The plot shows the initial rate of NADPH oxidation in the presence of 2 μM wild-type *Nc*CS or single mutant protein and 25 μM FMN as a function of NADPH concentration. Solid lines represent fits to the data points using a hyperbolic function giving K_m values of 9, 3, and 5 for wild-type *Nc*CS, the Asp367Ala mutant protein, and the Asp367Asn mutant protein, respectively.

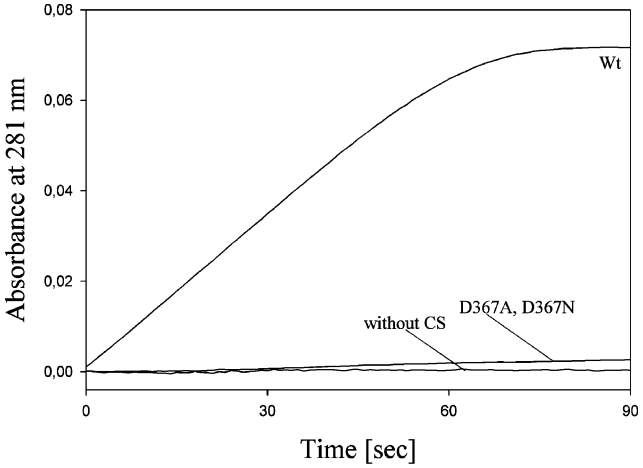


FIGURE 5: Activity of the aspartate mutant proteins Asp367Ala and Asp367Asn in comparison with that of wild-type (Wt) *Nc*CS. Spectrophotometric enzyme assay monitoring chorismate formation at 281 nm under aerobic conditions. The reactions were started by the addition of 30 μM EPSP to a mixture of 100 μM NADPH, 25 μM FMN, and 4 μM wild-type *Nc*CS or the Asp367Ala or Asp367Asn mutant protein. Reactions were carried out in 50 mM MOPS at pH 7.5 and 25 °C.

enzymes that can utilize NADPH directly to generate the required reduced cofactor, which is not consumed by substrate turnover (*4*, *15*). However, in the presence of dioxygen, the reduced cofactor undergoes spontaneous reoxidation, leading to inactivation of the enzyme. Under these conditions, the NADPH:FMN oxidoreductase activity of a bifunctional enzyme leads to the consumption of NADPH as demonstrated previously (*14*). This oxidoreductase activity was measured for both mutant proteins, as shown in Figure 4, yielding rates of NADPH consumption of 5 min^{-1}. This is approximately twice as fast as that measured for the wild-type enzyme (Figure 4). From the plots in Figure 4, K_M parameters of 3, 5, and 9 μM were deduced for the wild type, Asp367Ala, and Asp367Asn, respectively.

Taken together, these results demonstrate that the replacement of aspartate 367 with either alanine or asparagine neither affects the ability to bind FMN and EPSP nor impedes the utilization of NADPH as a source of reducing equivalents for activating the cofactor to its reduced form.

Chorismate Synthase Activity of the Mutant Proteins. The enzymatic activity of the mutant proteins was measured under two different experimental conditions. In a preliminary assessment of the activity, generation of chorismate was monitored under aerobic conditions in the spectrophotometer using NADPH as a source for reducing equivalents (*14*). This assay revealed that both the Asp367Ala and Asp367Asn mutant proteins had a very low residual activity of ≈1% of that of the wild-type enzyme (Figure 5). The activity was then also measured under anoxic conditions in the stopped-flow instrument. In the absence of oxygen, the rate of chorismate formation was 620 and 310 times slower for the Asp367Ala and Asp367Asn mutant proteins, respectively, than for the wild-type enzyme (Table 2), supporting our preliminary data reported above.

The chorismate synthase-catalyzed reaction is characterized by the occurrence of a transient species with a difference absorbance maximum at around 390 nm (*16*). This species

Table 2: Activity of the Aspartate Mutant Proteins Asp367Ala and Asp367Asn in Comparison with That of the Wild-Type Enzyme[a]

	wild-type *Nc*CS	Asp367Ala *Nc*CS	Asp367Asn *Nc*CS
k_{cat} (s^{-1})	0.87	0.0014	0.0028
%[b]	100	0.16	0.32

[a] Formation of chorismate at 275 nm was monitored using stopped-flow spectrophotometry under anaerobic conditions. After the samples had been mixed, the anaerobic reaction mixture contained FMN (80 μM), EPSP (100 μM), oxalate (1 mM), 50 mM MOPS (pH 7.5), and wild-type enzyme (1.25 μM) or aspartate mutant proteins (12.5 μM). [b] CS activity compared to wild-type *Nc*CS activity.

Table 3: Decay Rates of the Transient Flavin Intermediate[a]

	wild-type *Nc*CS	Asp367Ala *Nc*CS	Asp367Asn *Nc*CS
decay rate (s^{-1})	2.4	0.0042	0.0018
%[b]	100	0.18	0.08

[a] The decay rates for wild-type (Wt) *Nc*CS and the two mutant proteins were obtained using stopped-flow spectrophotometry (single-turnover). The absorbance changes were observed at 390 nm as a function of time. After the samples had been mixed, the anaerobic reaction mixture contained enzyme (20 μM), FMN (80 μM), EPSP (15 μM), oxalate (1 mM), and 50 mM MOPS (pH 7.5). [b] CS decay rates compared to the wild-type *Nc*CS decay rate.

is known to form after the substrate binds (*9*) but before EPSP undergoes any chemistry (*17*, *18*). The formation phase of the intermediate with the two mutant enzymes was almost complete within the dead time of the instrument and indistinguishable from that of the wild type. The decay rate of the intermediate in a single-turnover experiment roughly reflected the rate of substrate turnover. The Asp367Ala and Asp367Asn mutant proteins exhibited 570- and 1300-fold lower rates, respectively, for the decay of this transient species in comparison to that of the wild-type enzyme (Table 3). This result again demonstrates that the exchange of aspartate 367 with either alanine or asparagine compromises the enzymatic activity of the protein.

On the other hand, the spectral characteristic of the transient flavin species is not significantly affected by the mutation. As shown in Figure 6, the spectral features of the

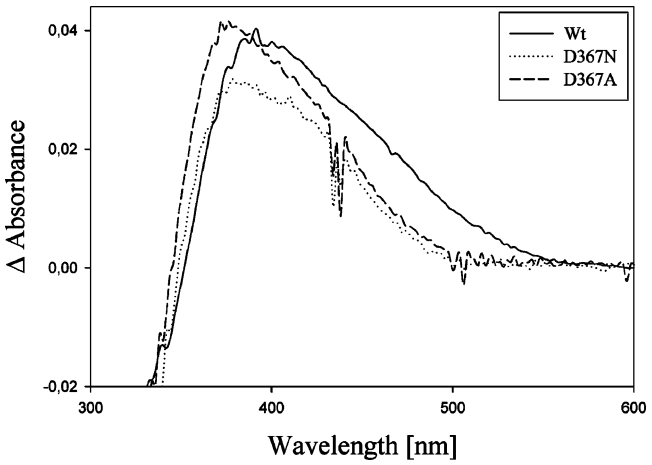


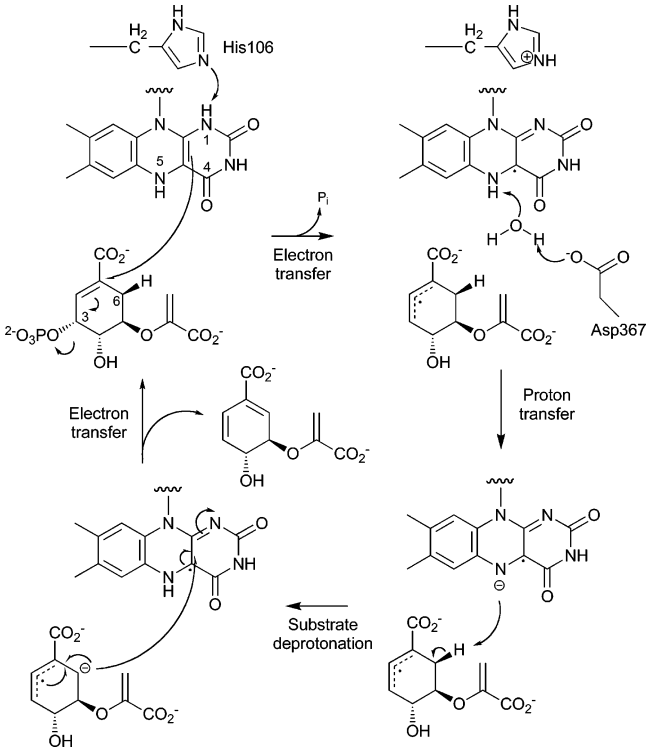
FIGURE 6: Observation of the flavin intermediate in the chorismate synthase reaction. Comparison of the difference absorbance spectra formed during the reaction with the wild-type enzyme (—), the Asp367Ala mutant protein (— — —), and the Asp367Asn mutant protein (···). The wild-type (Wt) trace was taken from Kitzing et al. (*12*). After the solution had been mixed, the anaerobic reaction mixture contained *Nc*CS (40 μM), reduced FMN (80 μM), EPSP (80 μM), oxalate (1 mM), and 50 mM MOPS (pH 7.5). Absorbance spectra were recorded between 300 and 600 nm. Each blank was obtained from control spectra in the presence of fully reduced FMN but in the absence of substrate.

intermediate are essentially conserved in both mutant proteins as compared to wild-type chorismate synthase.

DISCUSSION

The mechanism of the chorismate synthase-catalyzed reaction has provided several challenging issues revolving around the role of a reduced cofactor in a nonredox reaction and the mechanism that leads to an activation of the C(6*proR*) hydrogen with an estimated pK_a of ≈25 (*6*, *19*). Several kinetic studies have demonstrated that C−O bond cleavage precedes the abstraction of the C(6*proR*) hydrogen (*11*, *16*, *20*). Moreover, the results of these studies have pointed toward a radical mechanism in which the flavin donates an electron to the substrate to facilitate the C−O bond cleavage step (*8*, *11*, *21*). This concept was further supported by experimental studies using FMN analogues as active site probes for chorismate synthase (*9*). Theoretical studies have also indicated a transfer of charge from the reduced cofactor to EPSP, leading to C−O bond cleavage (*8*). From these mechanistic studies, a radical mechanism was favored for the chorismate synthase reaction with the reduced flavin, initiating phosphate cleavage leading to a substrate-derived radical species. However, the following steps involving the abstraction of the C(6*proR*) hydrogen remained obscure. The determination of the structure of *S. pneumoniae* chorismate synthase in the presence of oxidized FMN and EPSP provided the first insight into the binding and relative orientation of the cofactor and substrate in the active site of the enzyme (*10*). The structure is in keeping with the proposed role of reduced FMN as outlined above and also reveals the position of several invariant amino acid residues near the flavin ring system as well as the substrate. We recently demonstrated that the two conserved histidine residues function as general acids in the active site with H106 protonating the N(1)−C(2)═O locus of reduced flavin, whereas H17 appears to be involved in the protonation of

Scheme 2



the leaving phosphate group (*12*). Replacement of the histidines with alanine resulted in a modest decline in activity of factors of 10 and 20 for the H106A and H17A mutant proteins, respectively (*12*). The structure of the active site also suggests that aspartate 367 may have a role in the abstraction of the C(6*proR*) hydrogen mediated by a water-coupled deprotonation of the N(5) atom (Scheme 2). Hence, N(5) of the isoalloxazine ring may function as the long-sought acceptor in the second reaction step of the chorismate synthase-catalyzed reaction.

To test this hypothesis, we have conducted a mutagenesis study of Asp367 in which we have replaced this residue with alanine or asparagine. Our results clearly demonstrate that neither of these replacements compromises the ability of the enzyme to bind the cofactor or the substrate EPSP (Figures 2 and 3). Likewise, the bifunctionality of the mutant proteins is not affected by the change to alanine or asparagine (Figure 4). Similarly, both single mutant proteins show the occurrence of the transient flavin intermediate with similar spectral characteristics as observed in the reaction with the wild-type enzyme (Figure 6). However, both replacements reduce the activity of the mutant enzyme by factors of 620 and 310 for the Asp367Ala and Asp367Asn mutant proteins, respectively, clearly demonstrating the involvement of the carboxylate group in the catalytic reaction mechanism. This effect is much larger than previously observed for the two His106A and His17A single mutant proteins, emphasizing the importance of Asp367 in the reaction mechanism. Therefore, our results support a mechanism in which the carboxylate group of Asp367 participates in the deprotonation of the N(5) atom of the isoalloxazine ring system. The involvement of the carboxylate group may be mediated by a water molecule bound near Asp367 (see Figure 1) as outlined in Scheme 2 and proposed previously (*10*). However, it should be pointed out that a structure of chorismate

synthase in the presence of the reduced cofactor is not available, and hence, it is not known whether the carboxylate moves closer to the N(5) position at the expense of the bound water molecule, which may be expelled upon cofactor reduction.

The anionic flavin hydroquinone becomes protonated by His106 to give its neutral form when the substrate binds to the enzyme, and there is good evidence that the flavin intermediate observed in the stopped-flow spectrophotometer is this species (*9*). Initial transfer of an electron from the neutral hydroquinone to the substrate then leads to phosphate elimination and the formation of a flavin semiquinone (Scheme 2). The Asp367 residue must deprotonate the flavin N(5) position when the flavin is either fully reduced, as suggested by others (*10*), or in the semiquinone form. The former seems unlikely because deprotonation preferentially occurs at the N(1) position (with a pK_a of ~6.7) rather than at N(5) (*22*). Alternatively, it is possible that a cationic flavin is formed that has a pK_a of ~2.3. Although this species has chemical precedence (*23*), it has no precedence in flavin-dependent enzymatic reactions, and like the neutral hydroquinone, it preferentially loses a proton from N(1) rather than N(5). It is more likely that His106 abstracts a proton from N(1) concomitant with the initial transfer of an electron to give a neutral flavin semiquinone as shown in Scheme 2 (*12*). The crystal structure of the ternary complex indicates that His106 moves away from the flavin upon formation of the complex (*10*), but it is possible that it moves back upon elimination of the phosphate (or only moved because the structure was with oxidized flavin). It is most likely that Asp367 abstracts a proton from N(5) of the neutral flavin semiquinone at this point in the catalytic cycle. This form of the flavin is known to deprotonate preferentially from the N(5) position. The pK_a of this species is ~8.3 (*23*), and that of an aspartate side chain in solution is ~3.9; therefore, the environment of the active site presumably tunes these values to favor the proton transfer. The anionic semiquinone is then able to abstract a proton from the substrate radical as proposed previously (*24*). Subsequent electron transfer completes the catalytic cycle. Of course, these last two proton and electron transfer steps could be coupled. The stabilization of a neutral flavin radical species has been documented for chorismate synthase (*21*), albeit not during normal substrate turnover, indicating that decay of the proposed flavin radical species (Scheme 2) is rapid compared to its rate of generation. The mutation of Asp367 might have been expected to allow initial electron transfer to proceed (Scheme 2) but retard the rate of proton transfer, allowing the accumulation of a blue neutral semiquinone species. However, no additional species were observed in the single-turnover experiment, suggesting that initial electron transfer and proton transfer (the first two steps in Scheme 2) are somehow coupled. It therefore seems that potentially damaging radical chemistry does not commence until the scene is set to complete all the radical chemistry steps.

In summary, our results present the first experimental evidence that an invariant aspartate residue in the active site of chorismate synthase plays a pivotal mechanistic role working in concert with the N(5) position of the cofactor to bring about the abstraction of the C(6*proR*) hydrogen of the substrate. Hence, the flavin plays an unusual role in the chorismate synthase reaction in that it carries out a hidden transient redox process and acts as a cryptic active site base that is dependent on the assistance of the carboxylate group of Asp367.

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