Mechanism of Chorismate Synthase

ROLE OF THE TWO INVARIANT HISTIDINE RESIDUES IN THE ACTIVE SITE*

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Chorismate synthase catalyzes the last step in the common shikimate pathway leading to aromatic compounds such as the aromatic amino acids. The reaction consists of the 1,4-anti-elimination of the 3-phosphate group and the C-(6proR) hydrogen from 5-enolpyruvylshikimate-3-phosphate to yield chorismate. Although this reaction does not involve a net redox change, the enzyme has an absolute requirement for reduced flavin mononucleotide, which is not consumed during the reaction. Two invariant histidine residues are found in the active site of the enzyme: His\(^{17}\) and His\(^{106}\). Using site-directed mutagenesis, both histidines were replaced by alanine, reducing the activity 10- and 20-fold in the H106A and H17A mutant protein, respectively. Based on the characterization of the two single mutant proteins, it is proposed that His\(^{106}\) serves to protonate the monanionic reduced FMN, whereas His\(^{17}\) protonates the leaving phosphate group of the substrate. An enzymatic reaction mechanism in keeping with the experimental results is presented.

Chorismate synthase catalyzes the last step in the shikimate pathway leading to the branch point metabolite chorismate, which is utilized in a number of enzymatic transformations toward the biosynthesis of aromatic compounds such as the aromatic amino acids tyrosine, phenylalanine, and tryptophan (for a recent review, see Ref. 1). This reaction involves an 1,4-anti-elimination of the 3-phosphate group and the C-(6proR) hydrogen from 5-enolpyruvylshikimate-3-phosphate (EPSP)

The role of His\(^{106}\) is less clear; however, recent studies have suggested that this residue protonates the C-O bond cleavage of chorismate. In contrast to the mode of flavin reduction, the two classes of chorismate synthases show apparent differences in the mechanism of the elimination reaction.

Multiple sequence alignments of chorismate synthases from bacterial, fungal, plant, and protozoan origin have revealed a number of invariant amino acid residues in monofunctional as well as bifunctional chorismate synthases, suggesting a pertinent role in catalysis in both classes of enzymes (12). Among these are two histidine residues, His\(^{17}\) and His\(^{106}\), in the bifunctional N. crassa enzyme, which are both found in the active site of chorismate synthase within 2.7 and 3 Å of the C(2) = O position of the flavin ring (His\(^{106}\)) and the substrate phosphate group (His\(^{17}\)), respectively, as shown in Scheme 2. The structural proximity of the imidazole ring of His\(^{17}\) to the substrate phosphate group as well as its suitable properties as an acid-base catalyst suggest that this residue protonates the leaving group upon C-O bond cleavage.

The role of His\(^{106}\) is less clear; however, recent studies have provided evidence that the monanionic form of reduced FMN (pK\(_a\) = 6.7 free in solution (13)) is protonated upon binding of EPSP to the active site. Thus, His\(^{106}\) may be the general acid that protonates the N(1)-C(2) = O locus of the isooloxazine moiety. The rationale behind the protonation of the reduced flavin is the assumption that a neutral reduced flavin has a more negative redox potential and therefore may promote the initial electron transfer to the substrate more readily.

Another remarkable feature of the active site is the lack of an amino acid base in the vicinity of the C-(6proR) hydrogen. Instead, the flavin N(5)-position is close to this hydrogen, ar-

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The abbreviations used are: EPSP, 5-enolpyruvylshikimate 3-phosphate; DEPC, diethyl pyrocarbonate; MOPS, 3-(N-morpholino)propane-sulfonic acid; NcCS, N. crassa chorismate synthase; HOMO, highest occupied molecular orbital.
MATERIALS AND METHODS

Reagents—All chemicals were of the highest grade available and obtained from Sigma or Fluka (Buchs, Switzerland). DEAE Sephadex was from Amersham Biosciences, and cellulose phosphate (P11) was from Whatman (Kent, UK). DNA restriction and modification enzymes were obtained from Roche Molecular Biochemicals or New England Biolabs (Beverly, MA). PCR primers were from Microsynth (Balgach, Switzerland). EPSP was synthesized from shikimate-3-phosphate using a commercial enzyme (Biolabs, Beverly, MA). PCR primers were from Microsynth (Balgach, Switzerland). DNA restriction and modification enzymes was from Amersham Biosciences, and cellulose phosphate (P11) was obtained from Sigma or Fluka (Buchs, Switzerland). DEAE Sephacel was from Whatman (Kent, UK). DNA restriction and modification enzymes were obtained from Roche Molecular Biochemicals or New England Biolabs (Beverly, MA). PCR primers were from Microsynth (Balgach, Switzerland).

Purification of Neurospora crassa Chorismate Synthase (NcCS)—Both histidine to alanine mutant proteins were purified as described for wild-type protein (15). Yields and stability of the H17A and H106A mutant proteins were comparable with wild-type NcCS.

Enzyme Assays—A continuous enzyme assay was employed as described previously (15). Phosphate release from EPSP as a measure of enzyme activity was determined using a colorimetric assay as described for wild-type enzyme (15).

Site-directed Mutagenesis—Amino acid replacements were performed using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The construct pET21a-NcCS served as the template. The following oligonucleotides containing the appropriate codon for the desired amino acid were used for the procedure (the changed codon is underlined): H17A, forward primer, 5′-CGACCTATGGCGAGTCGGCCTGC-3′, and reverse primer, 5′-TCGAGGTAGGCTGCAGCCGGCCGGGTAGTGG-3′; H106A, forward primer, 5′-CATCTAACCCTCGCATGGACCTACGTGGAGC-3′, and reverse primer, 5′-TCGAGGTAGGCTGCAGCCGGCCGGGTAGTGG-3′.

All manipulations were performed following the manufacturer’s instructions. The mutations were verified by DNA sequencing (Microsynth). UV-visible Absorbance Spectrophotometry—Absorbance spectra were recorded with a Uvikon 933 double-beam spectrophotometer (Kontron Instrument AG, Zurich, Switzerland).

Fluorescence Spectroscopy—Fluorescence emission spectra were monitored with a Kontron spectrofluorimeter (model SFM-25). The excitation wavelength for tryptophan fluorescence was 280 nm, and for flavin fluorescence, it was 365 nm. All spectra were recorded in 50 mM MOPS, pH 7.5, at 25 °C.

Stopped-flow Spectrophotometry—Reaction kinetics were performed using a stopped flow spectrophotometer equipped with a thermostatted 1-cm path length cell and a diode array detector (SI Spectroscopy Instruments GmbH, Gutach, Germany). When necessary, enzyme and substrate solutions were made anaerobic by exchanging the dissolved oxygen with argon by several cycles of evacuation and flushing. Analysis of the data was performed using the program Spectfit32 (Dr. R. A. Binstead, Spectrum Software Associates, Marlborough, MA).

Redox Titrations—The redox potential of oxidized FMN/reduced FMN in the presence of wild-type NcCS and the two histidine mutant proteins was determined by the method described in Ref. 17. Details of the experimental conditions are given in Ref. 8. Anthraquinone-2,6-disulfonate (E° = −0.184 mV) was used as a reporter dye.

Molecular Orbital Calculations—Electron density calculations of reduced FMN in its N(1) protonated and deprotonated form were performed using the program MacSpartan, Version 1.1, from Wavefunction, Inc.

RESULTS

Inactivation of NcCS by Diethyl Pyrocarbonate (DEPC)—The imidazole ring of accessible histidine residues reacts specifically with DEPC, incapacitating its function as an acid-base catalyst (18). Incubation of NcCS with DEPC resulted in a time- (data not shown) and concentration-dependent inactivation of enzyme activity (Fig. 1). The enzyme activity of NcCS was completely inhibited by a 60-fold excess of DEPC after 1 h of incubation. Tryptic digestion followed by matrix-assisted laser desorption/ionization-time of flight mass analysis revealed that several histidine residues were labeled following incubation with DEPC (data not shown). Among the labeled histidine residues were the two invariant histidine residues His17 and His106 (numbering according to the N. crassa chorismate synthase sequence). Although it is not feasible to assign the inactivation of the enzyme specifically to one of the modified invariant histidines, the fact that both of these histidines are located in the active site and that DEPC inactivates the enzyme gave a first indication that the two invariant histidines play an important role in catalysis.

Site-directed Mutagenesis, Expression, and Purification of an H17A and H106A Single Mutant Protein—Both invariant histidine residues (His17 and His106) were replaced by alanine residues using site-directed mutagenesis. The two single histidine mutant proteins (H17A and H106A) were expressed and purified from crude extracts in a two-step chromatographic procedure as described for wild-type enzyme (15). Both mutant proteins exhibited similar expression levels and were successfully purified by the method developed for wild-type protein (15). Yields and stability of the H17A and H106A mutant proteins were comparable with wild-type NcCS.
Activity of the H17A and H106A Mutant Protein—Both histidine mutant proteins were first tested for activity in a continuous enzyme assay under aerobic conditions (Fig. 2A). In this assay, the activity of the H17A and H106A mutant protein was 0.1 and 0.25%, respectively, as compared with wild-type enzyme. Since the continuous assay is based on the intrinsic FMN:NADPH-oxidoreductase activity of NcCS (bifunctionality), which may also be affected by the mutation, we also determined the activity in an anaerobic assay where FMN was reduced prior to mixing with EPSP; thus, chorismate synthase activity can be determined independently from the oxidoreductase activity. The formation of chorismate was monitored spectrophotometrically at 300 nm in a stopped-flow apparatus (Fig. 2B). Under these conditions, the H17A and H106A mutant protein showed 5 and 10% of the wild-type enzyme activity, respectively. The higher activities of both mutant proteins in the absence of dioxygen indicate that the intrinsic oxidoreductase activity is also affected by the histidine to alanine replacement.

To check whether C–O bond breakage (phosphate elimination) is affected by the amino acid replacement, a third assay was employed that measures the release of phosphate from EPSP (Fig. 2C). As before, the activity decreased in the order wild-type > H106A > H17A with the same relative ratio between the two histidine mutant proteins. As phosphate release occurs early during catalysis and before C-(6 proR) hydrogen abstraction (6), this result indicates that both histidine residues are involved in chemical steps leading directly to the initial C–O bond cleavage. It should be noted that the reaction with wild-type enzyme is well in the range of saturation at the experimental conditions used, and thus, the activity of the mutant proteins is overestimated by a factor of 2–3-fold for the H106A and H17A mutant protein, respectively.

FMN:NADPH-oxidoreductase Activity of the Mutant Proteins—In contrast to the monofunctional chorismate synthases from bacterial and plant sources, fungal chorismate synthases have an intrinsic “secondary” activity in that they can utilize NADPH to reduce FMN to its catalytically active reduced form (bifunctionality) (19, 20). As the activity of the two histidine mutant proteins was higher in the anaerobic (Fig. 2B) than in the aerobic assay (Fig. 2A), an additional effect of the amino acid replacements on the capacity to use NADPH was indicated. This was confirmed by measuring the rate of NADPH oxidation in the presence of oxygen and absence of EPSP (Fig. 3). Under these conditions, both histidine mutant proteins showed a lower rate of NADPH oxidation amounting to 90 and 30% for the H17A and H106A mutant protein, respectively, as compared with wild-type enzyme (Fig. 3). $K_m$ values for NADPH were also affected by the mutation, increasing from 43 $\mu$M for wild-type NcCS to 110 and 280 $\mu$M for the H17A and H106A mutant protein, respectively.

In previously reported experiments, we have provided evidence that NADPH and EPSP compete for the same binding site. As a consequence, high concentrations of EPSP cause a decrease in the rate of NADPH oxidation (15). This inhibitory effect of EPSP is relieved upon conversion of EPSP to chorismate, resulting in a pronounced acceleration of NADPH oxidation (Fig. 4B, wild-type). As the two histidine mutant proteins are basically inactive under aerobic conditions (Fig. 2A), they cannot remove EPSP through conversion to chorismate, and thus, the inhibitory effect of EPSP remains constant in contrast to wild-type enzyme (Fig. 4B).

Binding of Oxidized FMN to the Histidine Mutant Proteins—Binding of oxidized FMN to both histidine mutant proteins was determined by difference UV-visible spectrophotometry and ultrafiltration assays as described recently (15). Both histidine mutant proteins possess dissociation constants similar to wild-type protein (Table I) suggesting that neither histidine residue is involved directly in binding of the oxidized FMN.

Moreover, the spectral changes observed upon binding of oxidized FMN to the histidine mutant proteins were identical to those seen with wild-type enzyme, indicating that the mutation does not affect the environment of the flavin in the active site (Fig. 5). Even in the presence of EPSP, binding of oxidized FMN to the histidine mutant proteins is unchanged (Table I), implying that neither of the two residues plays a role in binding of FMN in the ternary complex.

Binding of EPSP to the Histidine Mutant Proteins in the Presence of Oxidized FMN—Binding of EPSP to the H17A and H106A mutant protein in the presence of FMN was first monitored by UV-visible spectrophotometry. The observed spectral changes were used to calculate the dissociation constant for EPSP. In the case of the His$^{106}$ mutant protein, it is similar to the one determined for wild-type protein, whereas it is increased 5-fold for the H17A mutant protein (Table I and Fig. 6). This indicates that His$^{17}$ is involved in EPSP binding, whereas His$^{106}$ is not.

Qualitatively, the spectral changes observed when titrating the H17A mutant protein with EPSP are very similar to those reported earlier with the wild-type protein (Fig. 6A). In contrast to the H17A mutant protein, the H106A mutant protein exhibited strikingly different spectral changes (Fig. 6B). This finding provides spectroscopic evidence that His$^{106}$ is in close proximity to the flavin ring system, whereas His$^{17}$ is not. This result contrasts to the binding of oxidized FMN alone, which is not affected by either histidine replacement (Fig. 5), suggesting that binding of EPSP affects the spatial arrangement in the active site such that the flavin ring system and the His$^{106}$ interact with each other in the presence of substrate.

These findings received further support from fluorescence quench experiments where flavin emission is measured as a function of EPSP concentration. Although the H17A mutant protein exhibited changes similar to those observed with wild-type enzyme, the H106A mutant protein completely failed to show this effect (Fig. 7, A and B), corroborating the findings obtained by UV-visible absorbance spectrophotometry.

Binding of Reduced FMN to NcCS—Since the fully reduced (dihydroquinone) form of FMN is the catalytically relevant form in the chorismate synthase catalyzed reaction, we have also investigated its binding to NcCS. It has been shown previously that the reduction of FMN by NcCS leads directly to the
fully reduced dihydroquinone form without the occurrence of a radical species (15). Reduction of the flavin in the presence of a reporter dye of known redox potential allows the determination of the redox potential for the oxidized FMN/reduced FMN redox couple using UV-visible absorbance spectrophotometry (8, 17). In the presence of stoichiometric concentrations of wild-type NcCS, the redox potential of the FMN$_{ox}$/FMN$_{red}$ couple was determined to $-167$ mV, i.e. 40 mV more positive than that of the free couple in solution ($-207$ mV, (21)). This indicates a 23-fold tighter binding of the reduced form to wild-type enzyme as compared with oxidized FMN, amounting to a stabilization of 1.85 kcal/mol or a $K_d$ for reduced FMN of $1.7 \mu M$ ($39 \mu M$ for oxidized FMN (15)).
Observation of a Transient Flavin Intermediate—When an aerobic solutions of chorismate synthase-bound reduced FMN and EPSP are mixed in the stopped-flow instrument, a characteristic flavin-intermediate is transiently formed (6, 15, 22, 23). This species is generated very rapidly within a few milliseconds and disappears when all substrate has been consumed. Although the process leading to this intermediate is thought to be the protonation of the N(1) position of the anionic, reduced form of FMN ($pK_a$ of N(1)-H = 6.7 in free solution), its exact role in catalysis is not fully understood. To evaluate the effect of the histidine residues 17 and 106, both histidine mutant proteins were investigated with regard to their ability to form this typical flavin-derived intermediate. As shown in Fig. 8, the intermediate was observed with both histidine mutant proteins. With the H17A mutant protein, the intermediate is similar to the one observed with wild-type enzyme, having an absorbance maximum at 390 nm with a long wavelength absorbance in the range of 400–550 nm, albeit much less pronounced as for wild-type enzyme (Fig. 8B).

In contrast, the spectrum for the H106A mutant protein showed, in addition to the peak at 390 nm, a peak at shorter wavelength at 370 nm but hardly any absorbance in the long wavelength range (Fig. 8C). Again, the decay of the intermediate is considerably slower than with wild type and corresponds to the diminished rate of product formation (Fig. 2B). Thus, these observations show that both histidine residues affect the spectral characteristics and the lifetime of the transient flavin intermediate, with the H106A mutant protein exhibiting the most fundamental spectral changes in terms of absorbance maxima and the long wavelength band.

Molecular Orbital Calculations of Reduced Lumiflavin—The proposed protonation of the N(1) position of the anionic, reduced flavin in the process, leading to the formation of the transient flavin intermediate observed during the chorismate synthase catalyzed reaction, raises the following question: Why would this be required for efficient catalysis? To investigate this question, we were interested in the effect of N(1)-protonation on the electron distribution in the isalloxazine ring. Earlier quantum mechanical calculations have shown that deprotonation of N(1) in the reduced form have different effects on the atoms in the ring; although N(1) and the carbonyl oxygens gain negative charge, C(2), C(5a), N(5), and C(10a) lose electron density (24). A re-investigation of the electron density distribution in the HOMO of reduced lumiflavin (with a methyl group in position 10 instead of a ribitylphosphate side chain) confirmed this differential effect of the protonation state of N(1) on the electron distribution in the isalloxazine ring system. As shown in Fig. 9, protonation of N(1) causes a shift of electron density from the N(1) position (Fig. 9A) to C(10a), N(5), and C(4a) of the pyrazine ring. In view of the possibility that a transient electron transfer from reduced FMN to EPSP initiates C–O bond breakage, the location of the highest electron density in the isalloxazine ring may be a decisive factor in catalysis.

**DISCUSSION**

The conversion of 5-enolpyruvylshikimate 3-phosphate to chorismate by chorismate synthase is certainly one of the most intriguing flavin-dependent reactions in nature. Kinetic and mechanistic studies have accumulated substantial evidence for a radical mechanism in which the reduced, enzyme-bound FMN facilitates C–O bond cleavage by transient electron donation to the substrate (6, 7, 9, 25). The proposed radical chemistry for the reaction has been supported by theoretical considerations (26). Very recently, the elucidation of the first three-dimensional structure of the enzyme in complex with FMN and EPSP has shown that the substrate binds in close...
proximity on the si-face of the flavin isoalloxazine ring, clearly supporting a direct mechanistic role for FMN (10). This structure has also provided the first insight into the flavin environment and the amino acid residues involved in FMN and EPSP binding. A previous analysis of invariant amino acid residues has revealed the existence of two histidines (12), both of which are found in the active site of chorismate synthase (His17 and His106, Scheme 2). The histidine side chain with its pKa around 6 is a suitable and common catalyst for reactions requiring assistance by acid-base processes.

In our initial experiments with the histidine-specific reagent diethyl pyrocarbonate (DEPC), we confirmed that histidines are relevant for catalytic activity by chorismate synthase (Fig. 1). In conjunction with the structure and the invariance of His17 and His106 in all chorismate synthase sequences analyzed to date, this result suggests that these two active site histidines are indeed responsible for the observed deactivation by DEPC. To analyze the functional role of the two active site histidines in more detail, we have generated two single mutant proteins with an alanine replacement in either position 17 or position 106. Both single mutant proteins, H17A and H106A, showed a 10- and 20-fold diminished net activity for the elimination reaction in comparison with wild-type enzyme, respectively, corroborating the nearly complete inactivation by DEPC and demonstrating the importance of both histidines in the chorismate synthase reaction.

In the presence of oxygen, chorismate synthase activity is limited by reoxidation of the reduced cofactor. In the case of a bifunctional enzyme, NADPH can be utilized in "futile" cycles until all of the dissolved oxygen is removed and reduced FMN is no longer subject to reoxidation. The time course of this process depends on the NADPH:FMN oxidoreductase activity of the enzyme. Unexpectedly, both histidine to alanine replacements affected not only the chorismate synthase but also this intrinsic NADPH:FMN oxidoreductase activity. The rate of NADPH oxidation decreased in the order wild type/H11022 H17A/H11022 H106A with an accompanying increase in the Km for NADPH (2.5- and 6.5-fold, respectively). Although the effect on the rate of the oxidoreductase activity is comparatively small for the H17A mutant protein (90 versus 5%), the H106A mutant protein exhibits a more pronounced effect on the oxidoreductase relative to the chorismate synthase activity (30 versus 10%). This indicates that His106 also promotes the reduction of oxidized FMN in the active site of the enzyme, probably through

### Table I

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<tr>
<th>Ligand</th>
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<th>Method</th>
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<td>Ultrafiltration</td>
</tr>
<tr>
<td>FMN (in presence of EPSP)</td>
<td>16 ± 5a</td>
<td>UV/visible difference spectroscopy</td>
</tr>
<tr>
<td>EPSP (in presence of FMN)</td>
<td>12b</td>
<td>Ultrafiltration</td>
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a Values taken from Ref. 15.
b Average of three independent measurements.
c Average of four independent measurements.
d --, not determined.

**Fig. 5. Binding of oxidized FMN to the H106A mutant protein.** The plot shows titration of the H106A mutant protein (20 µM) with oxidized FMN in 50 mM MOPS buffer, pH 7. Arrows indicate the direction of the spectral changes occurring upon titration with FMN. Difference absorbance spectra at 0, 8.5, 16.9, 25, 33.1, and 56.3 µM oxidized FMN are shown. The inset shows a double reciprocal plot of the absorbance changes at 383 nm as a function of the FMN concentration, revealing a dissociation constant of Kd = 35 µM.
stabilization of the incipient negative charge on the reduced flavin (distance of NE2 to the carbonyl oxygen: 2.6–2.7 Å). This interpretation is supported by the observation that binding of reduced FMN is weaker in the mutant proteins, in particular in the H106A mutant protein. The less than 2-fold increase of the dissociation constant of reduced FMN to the H17A mutant protein suggests that this residue is also involved in its binding, albeit to a smaller extent.

Originally, the ability to utilize NADPH directly for the generation of the reduced FMN cofactor was thought to be
connected to a specialized nicotinamide binding fold in the bifunctional enzymes not existent in the monofunctional enzymes (19). However, recent biochemical studies have conclusively demonstrated that NADPH shares a common binding site with EPSP (15). In this context, it is also worth mentioning that the lack of activity of the two histidine to alanine mutant proteins under aerobic assay conditions (Fig. 4) does not lead to conversion of EPSP to chorismate and, as a result, the inhibitory effect of EPSP is not removed, as is the case with wild-type enzyme (Fig. 4B). This contributes to the diminished total activity of the bifunctional *N. crassa* chorismate synthase. Although both amino acid exchanges affect the intrinsic oxi-

![Fluorescence emission of oxidized FMN in the presence of NcCS as a function of EPSP.](http://www.jbc.org/)

**Fig. 7.** Fluorescence emission of oxidized FMN in the presence of NcCS as a function of EPSP. Flavin fluorescence was excited at 365 nm, and emission spectra were recorded between 400 and 700 nm. As shown in A, H17A mutant protein (10 μM) and FMN (12 μM) were titrated with EPSP. The fluorescence emission spectra shown were recorded at 0, 2.5, 5, 11, 24, 36, 97, 225, and 439 μM EPSP. Arrows indicate the direction of the fluorescence changes. The inset shows the changes in fluorescence emission at 525 nm as a function of EPSP concentration, giving a $K_d = 67 \mu M$. As shown in B, H106A mutant protein (10 μM) and FMN (12 μM) were titrated with EPSP. The fluorescence emission spectra shown were recorded at 0, 6, 78, and 138 μM EPSP.
Two Invariant Histidine Residues in Chorismate Synthase

A: N(1)-deprotonated reduced lumiflavin

B: N(1)-protonated reduced lumiflavin

Fig. 9. Molecular orbital calculation. An electron density representation of the HOMO of reduced lumiflavin (N(10)-methylisalloxazine) in its N(1)-deprotonated (A) and protonated (B) form is shown. The electron density is color-coded and decreases in the order blue > green > yellow > orange > red.

Recent studies have shown that the formation of the ternary protein complex consisting of chorismate synthase, FMN, and EPSP is associated with distinct UV-visible absorbance and fluorescence emission changes and were documented for monofunctional (8, 22) as well as for a bifunctional chorismate synthase (15). Thus, it can be concluded that these spectroscopic changes reflect the generation of a specific flavin environment in all chorismate synthases. In contrast to the H17A mutant protein, the absorbance and the fluorescence changes are completely abolished in the H106A mutant protein (Figs. 6 and 7), indicating that it is the interaction of the flavin ring with this amino acid side chain that is responsible for the observable spectroscopic effects. On the other hand, EPSP binding is only slightly affected by the H106A replacement, whereas the H17A mutant protein shows a 5-fold weaker binding of EPSP (Figs. 6 and 7). In the structure of the ternary protein complex, NE2 of His17 is within hydrogen bond distance to the phosphate group of EPSP, whereas NE2 of His106 is found in a similar distance to the N(1)-C(2) of FMN and the C(1)-carboxyl group of EPSP (Scheme 2) (10). Therefore, it can be concluded that neither of the latter two interactions plays a significant role in binding the oxidized FMN or EPSP. This is in contrast to His17, which provides binding energy for EPSP binding.

Clearly, the major difference between wild-type NcCS and the H106A mutant protein develops upon formation of the ternary complex, i.e. upon binding of EPSP to the active site. Interestingly, the relative orientation and the distance between His106 and the N(1)-C(2) of the flavin does not change much upon binding of EPSP (the distance of NE2 to C(2)-carbonyl oxygen is 2.68 and 2.56 Å in the binary and ternary complex, respectively (10)). Thus, it must be concluded that the observed spectral changes are a direct consequence of physical changes (e.g. polarity) introduced to this region upon EPSP binding to the si-face of the isoalloxazine ring. The most likely candidate for such an effect is the C(1) carboxyl group, which is positioned above the N(1)-C(2) = O locus of the flavin and within 3 Å of the histidine nitrogen (10). Although the effect on oxidized FMN is irrelevant for catalysis, previous studies with FMN derivatives, some of which are considered to be mimics of reduced FMN (7), have demonstrated preferential binding of the protonated, reduced FMN species rather than the N(1)-deprotonated species (pK_a = 6.7 free in solution (13)). This indicates that upon formation of a ternary protein complex (in this case with reduced FMN), the negatively charged C(1)-carboxylate triggers the protonation of N(1) by causing an increase of its pK_a. In this case, the protonation of N(1) is accompanied by a decreased absorbance change at 450 nm (10).
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scenario, His\textsuperscript{106} (pK\textsubscript{a} = 6.1 free in solution) functions as the acid that donates the proton to N(1). This role of His\textsuperscript{106} is supported by the pronounced spectral changes observed for the flavin intermediate in the case of the H106A mutant protein, which basically lacks the typical long wavelength absorbance and exhibits altered absorbance maxima (Fig. 8, C and D).

Although His\textsuperscript{106} may fulfill the role of a general acid protonating the monoanionic reduced FMN, this does not answer the most pertinent question: Why should this be required for catalysis? In earlier studies, we have concluded that the stabilization of the neutral reduced flavin species in the active site of chorismate synthase may be a reflection of an apolar flavin environment that in turn serves to lower the redox potential of the cofactor so as to render it a better reductant for a putative transient electron transfer to the substrate (7). However, in light of the three-dimensional structure with the imidazole ring of His\textsuperscript{106} and the C(1)-carboxylate group of the substrate near the N(1)-O locus of the flavin, this explanation does not appear to be plausible. On the other hand, the protonation status at N(1) impacts on the electron distribution in the HOMO orbital of the isoxazoline ring as shown by Ref. 24 and visualized in Fig. 9. Thus, protonation of N(1) may serve to direct the electron density so that charge/electron transfer to the double bond of the substrate is maximized. In other words, the substrate-induced pK\textsubscript{a} shift at N(1) tunes the electron density in the flavin ring so as to ensure efficient catalysis in a process termed earlier “substrate-induced cofactor activation” (7). Based on the structure and our findings with the two histidine mutant proteins described here, a more detailed mechanistic proposal arises (Scheme 3).

In this model, after binding of EPSP to the enzyme-bound reduced FMN (step I), an electron is transferred to the substrate double bond, initiating C–O bond cleavage and the release of phosphate with His\textsuperscript{17} acting as a general acid to neutralize the incipient charge on the oxygen atom (step II). The resulting C(4a)-neutral flavin semiquinone tautomerizes to a radical species where the unpaired electron resides on N(5) (step III) with concomitant abstraction of the proR-H from C(6) by the N(5) (step IV). This step is supported by the structure of the ternary complex, which shows that N(5) is the only candidate that can be invoked for the abstraction of the C(6(proR) hydrogen (distance N(5)-C(6(proR) hydrogen, –3 Å). In the final step, deprotonation of the reduced flavin restores the initial state of the cofactor (step V). Although this model depicts the crucial step II as transfer of an electron, it is also conceivable that a charge transfer interaction would suffice to drive the reaction. This has been postulated by Ref. 26 and is entirely consistent with the failure to detect a flavin radical species during catalytic turnover.

Future research efforts to understand this unprecedented (reduced) flavin-dependent reaction mechanism are bound to focus on the role of other invariant amino acid residues in the active site such as the two serine residues (17 and 127) that appear to function in the binding of a water molecule close to the oxygen bound to C(3) of EPSP, suggesting that stabilization of the phosphate leaving group is a governing factor in catalysis. Furthermore, the role of the carboxyl group at C(1) of EPSP also appears to be of some importance to the reaction mechanism. The contribution of these groups can be studied by site-directed mutagenesis and the utilization of substrate analogs, respectively, and will provide further insights into the mechanism of chorismate synthase.

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Mechanism of Chorismate Synthase: ROLE OF THE TWO INVARIANT HISTIDINE RESIDUES IN THE ACTIVE SITE
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