Spectroscopic and Kinetic Characterization of the Bifunctional Chorismate Synthase from *Neurospora crassa*

**EVIDENCE FOR A COMMON BINDING SITE FOR 5-ENOLPYRUVYLSHIKIMATE 3-PHOSPHATE AND NADPH**

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Chorismate synthase catalyzes the *anti*-1,4-elimination of the phosphate group and the C-(6proR) hydrogen from 5-enolpyruvylshikimate 3-phosphate to yield chorismate, a central building block in aromatic amino acid biosynthesis. The enzyme has an absolute requirement for reduced FMN, which in the case of the fungal chorismate synthases is supplied by an intrinsic FMN: NADPH oxidoreductase activity, *i.e.* these enzymes have an additional catalytic activity. Therefore, these fungal enzymes have been termed “bifunctional.” We have cloned chorismate synthase from the common bread mold *Neurospora crassa*, expressed it heterologously in *Escherichia coli*, and purified it in a three-step purification procedure to homogeneity. Recombinant *N. crassa* chorismate synthase has a diaphorase activity, *i.e.* it catalyzes the reduction of oxidized FMN at the expense of NADPH. Using NADPH as a reductant, a reduced flavin intermediate was observed under single and multiple turnover conditions with spectral features similar to those reported for monofunctional chorismate synthases, thus demonstrating that the intermediate is common to the chorismate synthase-catalyzed reaction. Furthermore, multiple turnover experiments in the presence of oxygen have provided evidence that NADPH binds in or near the substrate (5-enolpyruvylshikimate 3-phosphate) binding site, suggesting that NADPH binding to bifunctional chorismate synthases is embedded in the general protein structure and a special NADPH binding domain is not required to generate the intrinsic oxidoreductase activity.

**The abbreviations used are:** EPSP, 5-enolpyruvylshikimate 3-phosphate; MOPS, 3-[(morpholino)propanesulfonic acid; NcCS, *N. crassa* chorismate synthase; PAGE, polyacrylamide gel electrophoresis.
**Bifunctional Chorismate Synthase from Neurospora crassa**

NADPH-dependent reduction of flavin is up to 5 orders of magnitude faster in the presence of the substrate. In contrast to reactions in which redox equivalents are consumed stoichiometrically during substrate turnover, the FMN cofactor of chorismate synthase remains in its active reduced form during EPSP consumption (7, 12). However, molecular oxygen inactivates the enzyme as it reacts with reduced flavin to yield the oxidized flavin and oxygen peroxide. Although exact rate constants for reoxidation of the enzyme-bound reduced FMN by molecular oxygen have not been determined, this process occurs rapidly in vitro (6). Because the redox state of the flavin cofactor is crucial to chorismate synthase activity, it can be envisaged that the redox balance in the cell may play a critical role in regulating the generation of chorismate and perhaps the flow through the shikimate pathway as a whole. However, the parameters that may affect the rate of reoxidation of the chorismate synthase-bound reduced FMN have not been studied experimentally.

The bifunctional chorismate synthase provides an opportunity to directly study the reduction of its flavin cofactor with the physiological redundant NADPH as well as to monitor the reoxidation of the flavin by molecular oxygen with the same enzyme. Therefore, we have inserted the cDNA of the previously cloned gene for N. crassa chorismate synthase (9) into a high copy expression plasmid that can direct the expression of large quantities of the enzyme, thus allowing an extensive biochemical characterization. In this paper we report on the expression and purification of the enzyme and give a detailed analysis of its spectral and kinetic properties.

**MATERIALS AND METHODS**

**Reagents—**All chemicals were of the highest grade available and obtained from Sigma or Fluka (Buchs, Switzerland). DEAE-Sephacel was from Amersham Pharmacia Biotech, and cellulose phosphate (P11) was from Whatman. DNA restriction and modification enzymes were obtained from Roche Molecular Biochemicals or New England Biolabs (Beverly, MA). Polymerase chain reaction primers were from MBI (Fermentas, Hanover, NY). 10 mM Tris-HCl, pH 7.5, containing 50 mM KCI, 10% (v/v) glycerol, 1.3 mM EDTA, and 0.4 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and then loaded onto a cellulose phosphate column (2.5 cm x 1 cm) was resuspended in 20 ml of buffer A (0.1 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol) and then loaded onto a DEAE-Sephacel column (2.5 cm x 16 cm). Polymerase chain reaction primers were from Microsynth (Balghac, Switzerland). EPSP was a generous gift of Prof. Dr. John Coggins, University of Glasgow, UK.

**Expression and Purification of Anthranilate Synthase—**The plasmid pCH11–11B containing the E. coli trpE gene coding for anthranilate synthase component I was a generous gift from Dr. P. Pouwels (University of Rijswijk, The Netherlands). This plasmid is a derivative of the pCH plasmid family containing two copies of trpE. Competent E. coli PC 15622 cells (ΔtrpA-E, tryptophan auxotroph) were transformed with pCH11–11B and subsequently cultured according to Hesington et al. (15) overnight at 37 °C. The cells were then harvested, and the wet cell paste was stored at 3 °C. Bacterial extracts were analyzed for their expression of anthranilate synthase using 10% SDS-PAGE.

**Enzyme Assays—**Anthranilate synthase activity was determined using the procedure of Ito et al. (14), except that 0.03 M ammonium sulfate was added to the reaction mixture as an amino group donor (15). Chorismate synthase activity was measured using forward coupling photometry with the reaction catalyzed by AS as described by Schaller et al. (16). The reaction buffer contained 0.1 mM potassium phosphate, pH 7.8, 0.5 mM MgSO4, 10 mM glutamine, 0.33 mM ammonium sulfuric acid, 1 mM dithiothreitol, 10 μM FMN, 80 μM EPSP, and 50 picokatal of recombinant chorismate synthase component I from E. coli. FMN was reduced either by the addition of 5 mM dithionite or 1 mM NADPH.

**SDS-PAGE and Western Blot Analysis—**Protein samples were separated by SDS-PAGE performed on 10% gels according to Laemmli (17). Gels were stained with Coomassie Brilliant Blue R-250 or alternatively blotted onto nitrocellulose membranes. For the immunodetection of NcCS, an antibody raised against Corydalis sempervirens chorismate synthase (described in 16) and affinity-purified against E. coli chorismate synthase was used.

**Site-directed Mutagenesis—**To change amino acid residue 98 from a methionine to a lysine, the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) was used. The construct pET21a-NcCS was served as the template. The following oligonucleotides containing the appropriate codon exchange (ATG — AAAG) were used for the procedure (the changed codon is underlined): 5′-GACTAGCTAAAAGAAGACAGAGACATCTACCCGCCCTC-3′ and 3′-AGGCCGCGGGTATGCTCCTCTACTTCCTTTCGACCTGACTGC-5′. All manipulations were performed after the manufacturer’s instructions. The mutation was verified with an
Expression and Purification of N. crassa Chorismate Synthase—Heterologous expression of NcCS in conventional E. coli BL21 (DE3) cells resulted in low yields of protein because of the usage of rare codons in the N. crassa cDNA (data not shown). Protein expression (Fig. 1) was greatly improved by using BL21 CodonPlus (DE3)-RP cells, which provide the required tRNAs for rare arginine and proline codons. The expression of NcCS in BL21 CodonPlus (DE3)-RP cells amounts to \(-5\%\) of the total protein in crude extracts (Fig. 1, panel A, lane labeled Wt, +). Western blot analysis of the crude protein extract with an antibody raised against C. sempervirens chorismate synthase (16) and affinity-purified against E. coli chorismate synthase resulted in two bands of \(-41\) and \(-46\) kDa apparent molecular mass, respectively (Fig. 1, panel B, Wt, +). N-terminal sequence analysis identified the 46-kDa band as the full-length NcCS, whereas the 41-kDa band corresponds to a truncated form of NcCS with the first 97 N-terminal amino acids missing. Presumably the ATG coding for methionine 98 serves as a fortuitous translation start, giving rise to \(-50\%\) of the total NcCS protein expression. A sequence alignment of all known chorismate synthases (data not shown) indicated that the methionine in this position is not conserved, but the majority of chorismate synthases feature a lysine in the corresponding position. Therefore, the ATG codon was replaced with AAG, introducing a lysine instead of the methionine in position 98. This manipulation of the cDNA resulted in complete abolishment of the expression of the truncated NcCS as is evident from Fig. 1 (panel B, M98K +) and allowed the exclusive expression of the full-length enzyme. The purified M98K mutant protein has a specific activity of 0.8 units/mg, similar to the value of 0.7 units/mg reported by Welch et al. (7) for wild-type enzyme. Because the introduced mutation had no effect on the specific activity of the enzyme, the M98K mutant protein was used in all experiments throughout this study and is referred to as NcCS. Expression of the protein in this manner greatly facilitated its purification, and a homogeneous preparation was obtained in a three-step protocol comprising ammonium sulfate precipitation of NcCS from the crude protein extract followed by DEAE-anion exchange chromatography and phosphocellulose chromatography in an adaptation of the method used by White et al. (12). The progress of purification and the purity of NcCS is documented in Fig. 2. Typically, 70–80 mg of recombinant NcCS was obtained from 25 g of wet cell paste.

**Fig. 1.** Comparison of wild-type and M98K chorismate synthase expression in E. coli BL21 CodonPlus (DE3)-RP cells. E. coli BL21 CodonPlus (DE3)-RP cells were transformed with a plasmid carrying wild-type N. crassa chorismate synthase (aroC, labeled Wt) or the mutant M98K aroC. Uninduced and induced (0.3 mM isopropyl-1-thio-\(\beta\)-D-galactopyranoside) cell cultures are indicated by a (-) and (+), respectively. Protein molecular mass standards are indicated in kDa on the left. Panel A, Coomassie Blue-stained SDS-polyacrylamide gel; panel B, Western blot analysis of the same samples as in panel A using an affinity-purified antibody against E. coli chorismate synthase.

ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA) using the fluorescent dideoxy chain termination method.

N-terminal Amino Acid Sequencing—E. coli crude extracts were separated on 10% SDS-PAGE. Proteins separated by electrophoresis were then transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The band of interest was excised and subjected to automated Edman degradation in an applied Biosystems 477A sequencer. The band of interest was excised and subjected to automated Edman degradation in an applied Biosystems 477A sequencer. The band of interest was excised and subjected to automated Edman degradation in an applied Biosystems 477A sequencer. The band of interest was excised and subjected to automated Edman degradation in an applied Biosystems 477A sequencer.

**Fig. 2.** Purification of recombinant N. crassa chorismate synthase. Fractions from sequential purification stages were analyzed by SDS-PAGE and visualized by Coomassie Blue staining. S, soluble supernatant of the bacterial crude extract (ca. 10 \(\mu\)g); AS, 50% ammonium sulfate precipitate (ca. 4 \(\mu\)g); DEAE, pooled fractions after DEAE-Sephalose chromatography (ca. 4 \(\mu\)g); PC, pooled fractions after cellulose phosphate chromatography (ca. 2 \(\mu\)g); protein molecular mass standards are indicated in kDa on the left.
The dissociation constant of oxidized FMN alone ($K_d = 92 \mu M$) decreased ca. 10-fold in the presence of EPSP (Table I). The effect of EPSP on the binding of oxidized flavin with the N. crassa chorismate synthase is ca. 100-fold less pronounced than with the E. coli enzyme (6).

**UV-Visible Absorbance Spectrophotometry of NcCS—Purified recombinant NcCS was completely devoid of bound FMN (data not shown), indicating weak binding of the cofactor (7). A similar finding has been reported for the enzyme from E. coli (6) and Thermotoga maritima (18). Yet binding of oxidized FMN to NcCS can be directly monitored by the difference UV-visible spectroscopy, the results of which are shown in Fig. 3, panel A. The observed spectral changes are characterized by a hypochromic effect on the flavin absorbance with a maximum at 378 nm and an isosbestic point at 340 nm. Titration of NcCS with oxidized FMN showed saturation at high FMN concentrations, and a hyperbolic fit to the data produced a dissociation constant of 39 $\mu M$ (Fig. 3, inset of panel A) for FMN binding. Similarly, stoichiometric amounts of NcCS (31 $\mu M$) and oxidized FMN (25 $\mu M$) were titrated with EPSP. As shown in Fig. 3, panel B, the difference spectra are very similar in the near UV range, between 300 and 400 nm, whereas marked differences are found between 400 and 500 nm in comparison with the titration in the absence of EPSP (Fig. 3, panel A). In this range, a hyperchromic effect at 485 nm is observed with an additional isosbestic point at 475 nm (Fig. 3, panel B). As for oxidized FMN, titration with EPSP showed saturation behavior with a hyperbolic fit to the data giving a dissociation constant of 9 $\mu M$ (see Fig. 3, inset of panel B). To facilitate direct comparison to binding studies carried out with the monofunctional chorismate synthases from E. coli (6) and T. maritima (18), absolute spectral changes were recorded in a separate experiment shown in Fig. 4. From such a comparison, it is evident that the UV-visible spectral changes are similar for all enzymes studied thus far (6, 18), in particular in the near UV range. As noted previously, the observed hypochromic shift of the near UV absorbance peak at 370 nm and the higher resolution of the peak at 450 nm are indicative of a more apolar flavin environment (6, 18). The spectral changes were utilized to determine dissociation constants of oxidized FMN and EPSP in the presence of oxidized FMN as shown in the insets of Figs. 3 and 4 and are summarized in Table I.

**Fluorescence Emission Quench Studies**—The effect of EPSP on the fluorescence emission of enzyme-bound flavin was also examined. As shown in Fig. 5A, the flavin fluorescence emission was quenched by EPSP and was characterized by a broadening of the emission maximum at 525 nm accompanied with an almost 2-fold decrease in fluorescence intensity. This quench of fluorescence intensity is associated with the emergence of a shoulder at ~450 nm (Fig. 5A). To observe changes in the protein directly during binding of flavin and EPSP, tryptophan fluorescence emission was also examined. NcCS possesses a single tryptophan residue at position 109, which is in the vicinity of a highly conserved sequence motif (R$^{108}$PGHAD$^{106}$). The addition of flavin to the protein results in

| Ligand | $K_d$ | Method |
|--------|-------|--------|
| EPSP alone | $33 \pm 12^a$ | Ultrafiltration |
| Oxidized FMN alone | $92 \pm 12^b$ | Ultrafiltration |
| EPSP (in the presence of oxidized FMN) | $39^c$ | UV-visible difference spectroscopy |
| | $17^d$ | Ultrafiltration |
| | $16^e$ | Trp fluorescence quenching |
| | $12^f$ | Flavin fluorescence quenching |
| | $17 \pm 4^g$ | UV-visible spectroscopy |
| | $9^h$ | UV-visible difference spectroscopy |
| Oxidized FMN (in the presence of EPSP) | $9 \pm 5^i$ | Ultrafiltration |

*Average of nine measurements.

$^b$ Average of three measurements.

$^c$ Average of two measurements.

$^d$ Average of five measurements.

$^e$ Average of nine measurements.

$^f$ Average of three measurements.

$^g$ Average of two measurements.
the solid line is a hyperbolic fit to the data. The fit indicated a dissociation constant of 17 \mu M.

Fig. 4. UV-visible spectral changes recorded during titration of NcCS and FMN with EPSP. Chorismate synthase (41 \mu M) and FMN (25 \mu M) in 50 mM MOPS, pH 7.5, were titrated with EPSP. The absorbance spectra shown were recorded between 300 and 600 nm at EPSP concentrations of 0, 2, 4, 7.9, 11.8, 23.2, 49, 121.7, and 331.4 \mu M, respectively. The arrows indicate the direction of the observed spectral changes. The inset shows the spectral changes at 383 nm as a function of the EPSP concentration. The circles represent the data points, and the solid line is the hyperbolic fit to the data. The fit indicated a dissociation constant of 17 \mu M.

Fig. 5. Binding of EPSP to NcCS in the presence of FMN monitored by fluorescence emission quenching. Stoichiometric amounts of chorismate synthase (10 \mu M) and FMN (12 \mu M) were titrated with EPSP, and the fluorescence emission quenching of the flavin fluorescence (panel A) and the tryptophan fluorescence (panel B) were monitored. Spectrum I in panel B shows the fluorescence emission spectrum of chorismate synthase alone, i.e. in the absence of FMN and EPSP. Starting with spectrum 2 (recorded after addition of FMN), EPSP was added to a final concentration of 2.5, 4.9, 9.8, 14.6, 19.3, 29, 48.2, 95.9, 142.8, 235, 368.7 \mu M, respectively (from top to bottom). Fluorescence emission was excited at 365 and 280 nm for FMN and tryptophan, respectively. The arrows in both panels indicate the direction of the observed fluorescence changes. The insets show the changes at 525 nm (panel A) or 353 nm (panel B) as a function of EPSP concentration. The data points are shown as solid circles, and the solid line was generated using the equation \Delta \lambda = ([FMN] \times \Delta \lambda_{max}[K_d + [FMN]]) + constant \times [FMN]. The K values obtained are shown in the insets.

Bifunctional Chorismate Synthase from Neurospora crassa
Bifunctional Chorismate Synthase from Neurospora crassa

FIG. 6. Reduction of NeCS with NADPH in the absence of oxygen. NeCS (7 μM) and FMN (25 μM) were incubated under anaerobic conditions. The reaction was initiated by the addition of 100 μM NADPH. Spectra were recorded between 300 and 550 nm at 2-min intervals (top to bottom as indicated by the arrows). The gray line shows the spectrum of oxidized flavin before the addition of NADPH. The dashed line shows the spectrum after reoxidation of the reduced flavin by incubation with oxygen.

TABLE II
Observed rates of FMN reduction as a function of 5-enolpyruvylshikimate 3-phosphate and NADPH

| Concentrations used: NeCS (20 μM) and FMN (20 μM). | ND, not determined. |
|---------------------------------|------------------|
| NADPH (μM) | EPSP (μM) | $k_{\text{red}}$ | $M^{-1} s^{-1} \times 10^3$ |
|---------|---------|-------------|------------------|
| 25      | 40      | 26          | ND               |
| 50      | 71      | ND          | 45               |
| 100     | ND      | ND          | 55               |
| 125     | ND      | ND          | 56               |
| 200     | ND      | ND          | 46               |
| 250     | ND      | ND          | 57               |
| 500     | 71      | 45          | 56               |

FIG. 7. Formation of a flavin intermediate during substrate turnover with NeCS. Before reaction with EPSP, FMN (40 μM) was reduced at the expense of NADPH (500 μM) in the presence of NeCS (40 μM) under anaerobic conditions. After completion of the reaction, reduced NeCS/FMN was mixed with EPSP in the stopped-flow instrument. After mixing, the final concentrations were 20 μM enzyme, 20 μM FMN, 250 μM NADPH, and varying concentrations of EPSP depending on the experiment. Panel A shows the absorbance differences at 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 15 s (from top to bottom) after the reaction was started (50 μM EPSP, single turnover), and Panel B shows a time course of spectra recorded at 0.1, 0.4, 7.4, 12.2, 19.6, and 34.5 s at an EPSP concentration of 500 μM (multiple turnover). Panel C shows the absorbance changes at 300 and 392 nm as a function of time using an EPSP concentration of 250 μM (multiple turnover).

TABLE III
Reoxidation of reduced FMN by molecular oxygen as a function of 5-enolpyruvylshikimate 3-phosphate

| Concentrations used: NeCS (25 μM), FMN (25 μM), and NADPH (25 μM). |
|-------------------|-------------------|
| EPSP (μM) | k_{\text{chs}} | k_{\text{reox}} |
| μM     | $s^{-1}$ | $M^{-1} s^{-1} \times 10^4$ |
| 0      | 1.5 ± 0.22 | 1.44 |
| 125    | 1.3 ± 0.26 | 1.25 |
| 500    | 2.0 ± 0.3  | 1.92 |

dithionite or photoreduction. Because NeCS utilizes the biologically relevant reductant NADPH (Fig. 6) to reduce the FMN cofactor, this system offers the unique opportunity to study the generation and occurrence of the flavin intermediate under more physiological conditions. Moreover, formation of the reaction intermediate has thus far only been demonstrated for monofunctional (18, 20) but not for bifunctional chorismate synthases. When NeCS-bound FMN is reduced by NADPH in the absence of molecular oxygen and mixed in a stopped-flow apparatus with EPSP, the intermediate is formed within the dead time of the instrument (ca. 5 ms) and is characterized by an absorbance maximum at 392 nm and a broad shoulder at longer wavelength (Fig. 7A). The spectral features of the intermediate are very similar to those reported with the enzymes from E. coli and T. maritima (18–20). The diode array for data acquisition allowed both chorismate formation ($\lambda_{\text{max, chorismate}} = 285 \text{ nm}$) and the presence of flavin intermediate (at 392 nm) to be monitored simultaneously in a single experiment as is shown in Fig. 7, panel B. A direct comparison of chorismate formation and the presence of the intermediate is shown in Fig. 7, panel C. From this plot, it is evident that the flavin intermediate (as represented by the trace at 392 nm) is present during substrate turnover/chorismate formation and decays when chorismate is fully formed (trace at 300 nm).

Consumption of NADPH, Formation of Chorismate, and Reduction of NeCS-bound FMN in the Presence of Molecular Oxygen—The data shown in Fig. 6 demonstrate the reduction of FMN by NADPH under anaerobic conditions. In the presence of oxygen (256 μM (21)), FMN (25 μM) reduction occurs in the presence of NADPH and NeCS; however, the reduced flavin is spontaneously reoxidized by molecular oxygen. Hence, under the conditions used here, NADPH is completely consumed by these two processes (Fig. 8A). Because this consumption of NADPH also occurs in the absence of EPSP, it can be concluded that the oxidoreductase activity is functionally independent of the chorismate synthase activity. Under aerobic conditions, the rate at which NADPH is consumed (Scheme 2) depends strongly on EPSP concentration (Fig. 8A). At very low EPSP concentrations (0–10 μM), there is no observable effect on the
rate of oxidation of NADPH (Fig. 8A). However, under the experimental conditions used, the apparent rate of NADPH oxidation appears maximal at 25 μM EPSP (6-fold excess over NcCS) (Fig. 8A). At higher EPSP concentrations, NADPH consumption is characterized by an initial lag phase followed by a fast decline in the NADPH concentration (Fig. 8A). The initial lag phase at higher EPSP concentrations corresponds to EPSP turnover as shown in Fig. 8B, indicating that the rate of NADPH oxidation is diminished during catalysis, i.e. EPSP and NADPH are competing for the same binding site. This competition is gradually relieved by consumption of EPSP, resulting in faster oxidation of NADPH, and gives rise to the complex time course of NADPH oxidation (Fig. 8A). The inhibition of NADPH oxidation (or FMN reduction) is also noticed in the rate of EPSP consumption because reduction of the flavin is required to activate the enzyme. Therefore, at high EPSP concentrations, the initial rate of EPSP consumption is reduced because of a lower concentration of reduced FMN/NcCS that in turn results from a competition for the common binding site (Fig. 8B, inset). Because the concentration of EPSP decreases, this competition is relieved, leading to more reduced FMN/NcCS and, hence, a faster rate of chorismate formation.

In parallel studies we have also investigated the reduction of FMN during NADPH and EPSP turnover in the presence of oxygen. As shown in Fig. 9, FMN reduction monitored at 450 nm shows a rapid initial phase followed by a steady-state level of reduced FMN. This approach to steady state is delayed at higher EPSP concentrations, again indicating that during EPSP turnover access to the FMN binding site is blocked by EPSP, resulting in an inhibitory effect on flavin reduction.

Development of a Convenient Aerobic Chorismate Synthase Assay—In the previous sections it has been demonstrated that chorismate formation takes place in the presence of molecular oxygen (256 μM at 25 °C), i.e. despite reoxidation (and hence inactivation) of the NcCS-bound reduced FMN cofactor. We have exploited this feature to establish a reproducible aerobic assay for the chorismate synthase reaction as shown in Fig. 10. At low NADPH concentrations, the chorismate synthase reaction cannot be sustained long enough to convert all EPSP to chorismate as monitored at 281 nm (isosbestic point for the oxidation of NADPH to NADP⁺). However, under the experimental conditions used, the apparent rate of NADPH oxidation appears maximal at 25 μM EPSP (6-fold excess over NcCS) (Fig. 8A). At higher EPSP concentrations, NADPH consumption is characterized by an initial lag phase followed by a fast decline in the NADPH concentration (Fig. 8A). The initial lag phase at higher EPSP concentrations corresponds to EPSP turnover as shown in Fig. 8B, indicating that the rate of NADPH oxidation is diminished during catalysis, i.e. EPSP and NADPH are competing for the same binding site. This competition is gradually relieved by consumption of EPSP, resulting in faster oxidation of NADPH, and gives rise to the complex time course of NADPH oxidation (Fig. 8A). The inhibition of NADPH oxidation (or FMN reduction) is also noticed in the rate of EPSP consumption because reduction of the flavin is required to activate the enzyme. Therefore, at high EPSP concentrations, the initial rate of EPSP consumption is reduced because of a lower concentration of reduced FMN/NcCS that in turn results from a competition for the common binding site (Fig. 8B, inset). Because the concentration of EPSP decreases, this competition is relieved, leading to more reduced FMN/NcCS and, hence, a faster rate of chorismate formation.

Fig. 8. Influence of EPSP on NADPH oxidation and chorismate formation catalyzed by NcCS. Panel A, effect of EPSP on the time course of NADPH (100 μM) oxidation as catalyzed by NcCS (4 μM) in the presence of FMN (25 μM) and molecular oxygen (256 μM) monitored at 340 nm. The reactions were started by addition of NADPH (100 μM) to the reaction mix (25 °C). The numbers next to the curves indicate the EPSP concentration (in μM). Panel B, experimental conditions as for panel A, but the reaction was followed at 281 nm (an isosbestic point for the oxidation of NADPH to NADP⁺). The inset shows the initial phase of the reaction.

Fig. 9. Steady-state analysis of FMN reduction in the presence of oxygen as a function of EPSP concentration. All reactions were carried out in a stopped-flow apparatus equilibrated at 25 °C. The reactions were started by mixing 100 μM NADPH, 256 μM molecular oxygen with chorismate synthase (20 μM), FMN (20 μM), and dioxgen (256 μM) as a function of EPSP concentration. The reduction of FMN was monitored at 450 nm.
ing a limiting rate at ca. 200 μM NADPH. This set-up provides a simple continuous method to measure bifunctional chorismate synthase activity without the requirement for time-consuming operations to exclude oxygen from the assay mixture.

**DISCUSSION**

The present study provides a detailed biochemical characterization of a bifunctional chorismate synthase and, in particular, of its intrinsic oxidoreductase activity. The enzyme from the bread mold *N. crassa* has been heterologously expressed in *E. coli* (Fig. 1), and a three-step purification protocol has been established to obtain amounts of homogeneous chorismate synthase sufficient for spectroscopic and kinetic studies (Fig. 2). The protein was isolated in its FMN-free form, indicating that oxidized FMN binds only weakly. Similar observations have been reported for chorismate synthases from *E. coli* (6) and *T. maritima* (18). In agreement with this observation, titration of the enzyme with FMN revealed a rather high dissociation constant in the range of 40–90 μM (Table I). Again, similar dissociation constants have been obtained for the *E. coli* and *T. maritima* enzymes (30 and 140 μM, respectively, (6, 18)). In the presence of the substrate EPSP, binding of oxidized FMN is ca. 5–10 times tighter (Table I). This effect of EPSP is not as pronounced as has been found for the *E. coli* enzyme, which showed a 1500-fold tighter binding of oxidized FMN in the presence of EPSP (6). Binding of oxidized FMN alone to NcCS is associated with spectral changes in the UV-visible difference absorbance spectrum of the flavin (Fig. 3A). These changes are characterized by a hypsochromic shift of the near UV band of the flavin absorbance spectrum and have been described for the well-characterized *E. coli* enzyme as well as for the *T. maritima* enzyme (6, 18). Further spectral changes occur upon the addition of EPSP to the long wavelength region around 480 nm (see Fig. 3, panel B), indicating that the flavin environment is altered in the presence of EPSP. These spectral changes have been interpreted in terms of a more hydrophobic environment in the FMN binding pocket (6, 22). This general interpretation was confirmed for the enzyme from *E. coli* (4), which produces an increase of pKₐ values for the ionizable groups of the flavin analogs 6-hydroxy- and 8-mercapto-FMN. This result has now been substantiated by fluorescence quenching of the NcCS-FMN complex during titration with EPSP. The blue shift of the flavin fluorescence maximum at 525 nm and the occurrence of an additional peak at 450 nm also suggest a more hydrophobic environment upon binding of EPSP to the binary NcCS-FMN complex (6). The hypothesis has been put forward that the hydrophobicity of the FMN binding pocket renders the reduced flavin a better electron donor for its assumed role as a transient electron transfer agent to EPSP to initiate cleavage of the C-O bond (4, 5). However, to date no flavin radical species has been observed spectroscopically during EPSP turnover. The only intermediate in the chorismate synthase-catalyzed reaction that could be observed is the N(1)-protonated form of reduced FMN, which forms rapidly upon binding of EPSP and prior to C-O and C-H bond cleavage (6, 19). Rapid reaction studies performed with NcCS now confirm the occurrence of this intermediate for a bifunctional chorismate synthase and emphasize the importance of this intermediate in catalysis for monofunctional as well as bifunctional chorismate synthases.

Our studies have also shown that the flavin intermediate occurs only during EPSP turnover and relaxes to the initial species, i.e. the N(1)-deprotonated reduced flavin species, after complete conversion of EPSP to chorismate. This is the first time that the presence of the flavin intermediate could be correlated to EPSP turnover in a single experiment; this strongly supports earlier parallel measurements with the *E. coli* enzyme (19).

The most salient property of NcCS is the utilization of NADPH to generate the essential reduced FMN cofactor, a characteristic feature of the bifunctional fungal chorismate synthases (Scheme 2). In the present work, we have shown that NADPH is consumed via reduction of FMN followed by reoxidation of the reduced FMN by molecular oxygen. During NADPH turnover in an aerobic solution, a steady-state concentration of reduced enzyme gives rise to EPSP conversion to chorismate. Therefore, NcCS is an appropriate system to study chorismate formation in an aerobic environment typically encountered *in vivo*.

The study of the reductive half-reaction (Scheme 2) revealed that the observed rate of flavin reduction appears to be independent of the NADPH concentration and the presence of EPSP. The first observation indicates that binding of NADPH and the transfer of redox equivalents from NADPH to oxidized flavin are not rate-limiting. In view of the weak binding of oxidized FMN, the most likely explanation of the slow and NADPH concentration-independent rate of reduction is that binding of oxidized FMN to NcCS is the rate-determining step in the overall reduction process. Earlier reports on the effect of EPSP on catalytic activity of *N. crassa* chorismate synthase were inconclusive because the role of FMN as an intrinsic factor of catalysis was poorly understood at that time (7, 23). Our studies clearly show that EPSP exerts a negligible effect on the rate of FMN reduction under anaerobic conditions (see Table II). This finding is in contrast to the rate of reduction of FAD-dependent hydroxylases, which was found to be 4–5 orders of magnitude faster in the presence of the aromatic substrate (10, 11). However, it should be pointed out that in this case NADPH is consumed stoichiometrically during the course of the enzyme-catalyzed redox reaction, whereas in the case of chorismate synthase, reduction of FMN is required only once to achieve multiple turnover of EPSP. Therefore, one could argue that substrate-induced rate enhancement of FMN reduction is not required in the case of bifunctional chorismate synthase.

Because reoxidation of NcCS-reduced FMN by molecular oxygen leads to inactivation of the enzyme, it could be limiting to chorismate formation under aerobic conditions. The rate of reoxidation measured for NcCS-bound reduced FMN is in the same range as observed for flavoprotein oxidases (ca. 1.5 × 10⁴ M⁻¹ s⁻¹ versus 0.9 × 10⁴ M⁻¹ s⁻¹ for l-lactate oxidase (24), 8.5 × 10⁴ M⁻¹ s⁻¹ for glycolate oxidase (25), and 6.2 × 10⁴ M⁻¹ s⁻¹ for l-aspartate oxidase (26)), which have evolved to transfer substrate-derived redox equivalents to molecular oxygen. In comparison with these reoxidation rates, it can be concluded that the NcCS-bound reduced FMN is readily accessible to molecular oxygen, and hence, protection of the reduced FMN does not occur. Nevertheless EPSP turnover occurs in the pres-
ence of oxygen (see Figs. 8B), indicating that it can effectively compete by molecular oxygen. This is clearly supported by the rates determined for EPSP turnover (1.3 s⁻¹ under anaerobic conditions) and the rate of reoxidation of the catalytically essential reduced FMN by dissolved oxygen (1.5 s⁻¹).

Perhaps the most intriguing question revolving around bifunctional chorismate synthases is the nature and location of the NADPH binding site. Initially, it was thought that bifunctional chorismate synthases possess an additional domain that is responsible for NADPH binding (9). This proposal was mainly based on the larger molecular mass of the bifunctional as compared with the monofunctional enzymes (ca. 4–8 kDa). Based on amino acid sequence alignments, Henstrand et al. (9) identified two regions in bifunctional enzymes that are absent in monofunctional enzymes, suggesting that these may be associated with NADPH binding. To test this hypothesis, several forms of the N. crassa enzyme lacking the areas in question were engineered (9). In all cases studied, utilization of NADPH as a source for redox equivalents was retained, indicating that none of the truncated regions are critical for NADPH binding. Hence, it can be concluded that the ability to bind NADPH is embedded in the chorismate synthase structure. In this context, it is noteworthy that the bifunctional chorismate synthases lack a typical dinucleotide Rossmann fold (27), indicating an unusual mode of interaction between these chorismate synthases and NADPH.

In the present study, it is documented that the rate of NADPH oxidation strongly depends on the EPSP concentration; although at low EPSP concentration (equimolar to 10-fold excess over enzyme) the rate of NADPH oxidation is enhanced, this rate decreases significantly at higher EPSP concentrations, giving rise to a pronounced lag phase. This lag phase also corresponds to slower EPSP consumption (Fig. 8B), indicating that EPSP and NADPH compete for a common binding site. Because the rate of flavin reduction and reoxidation by molecular oxygen was found to be independent of the EPSP concentration in single turnover experiments (Table III), it must be concluded that this is not the case under multiple turnover conditions. In fact, under multiple turnover conditions and at low EPSP concentration (equimolar to 2.5-fold excess over enzyme), the initial reduction of FMN is faster (approaching steady state) and reaches a higher level of reduced FMN during steady state (Fig. 9). Hence, it appears that low EPSP concentrations have a stimulating effect on NADPH oxidation/FMN reduction. Because both NADPH and EPSP possess a phosphate group, it is conceivable that this group plays an important role in binding to the active site of chorismate synthase. The lack of a three-dimensional structure of chorismate synthase does not allow a more detailed discussion of this issue, but it should be noted that the fungal bifunctional chorismate synthases (N. crassa, S. cerevisiae, and Schizosaccharomyces pombe) feature several conserved basic amino acid residues absent in monofunctional enzymes. These residues may provide the correct spatial interactions to the phosphate group of NADPH necessary to achieve effective binding to the active site of bifunctional chorismate synthases.

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