Evidence for a major structural change in Escherichia coli chorismate synthase induced by flavin and substrate binding

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Chorismate synthase (EC 4.6.1.4) catalyses the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP) into chorismate, and requires reduced FMN as a cofactor. The enzyme can bind first oxidized FMN and then EPSP to form a stable ternary complex which does not undergo turnover. This complex can be considered to be a model of the ternary complex between enzyme, EPSP and reduced FMN immediately before catalysis commences. It is shown that the binding of oxidized FMN and EPSP to chorismate synthase affects the properties and structure of the protein. Changes in small-angle X-ray scattering data, decreased susceptibility to tryptic digestion and altered Fourier-transform (FT)-IR spectra provide the first strong evidence for major structural changes in the protein. The tetrameric enzyme undergoes correlated screw movements leading to a more overall compact shape, with no change in oligomerization state. The changes in the FT-IR spectrum appear to reflect changes in the environment of the secondary-structural elements rather than alterations in their distribution, because the far-UV CD spectrum changes very little. Changes in the mobility of the protein during non-denaturing PAGE indicate that the ternary complex may exhibit less conformational flexibility than the apoprotein. Increased enzyme solubility and decreased tryptophan fluorescence are discussed in the light of the observed structural changes. The secondary structure of the enzyme was investigated using far-UV CD spectroscopy, and the tertiary structure was predicted to be an α-β-barrel using discrete state-space modelling.

INTRODUCTION

Chorismate synthase (EC 4.6.1.4), the seventh enzyme of the shikimate pathway, catalyses the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP) into chorismate (Scheme 1). Being present in bacteria and fungi, but not in mammals, this enzyme is an attractive target for new anti-microbial agents. This promise is realized by the discovery of a functional enzyme in bacteria and fungi, but not in mammals, this enzyme is an attractive target for new anti-microbial agents. This promise is realized by the discovery of a functional enzyme...
chorismate synthase affects the properties and structure of the protein. Changes in small-angle X-ray scattering data, decreased susceptibility to proteolytic digestion and altered Fourier-transform (FT)-IR spectra provide the first strong evidence for major structural changes in the protein. Increased mobility of the protein in non-denaturing PAGE, increased enzyme solubility, decreased tryptophan fluorescence, little change in far-UV CD spectra and secondary structure evaluations and predictions are discussed in the light of the observed structural changes.

EXPERIMENTAL

Materials

Chorismate synthase was purified from an overproducing strain of Escherichia coli (AB2849/pGM605), as described previously [13]. From 20 g of cell paste, 125 mg of enzyme was obtained with a purity of > 95 %, as judged by SDS/PAGE. The enzyme was stored in bead form in liquid nitrogen. FMN (approx. 95 %) was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), and the potassium salt of EPSP was prepared as described by Knowles et al. [14].

Fluorescence emission measurements

Fluorescence emission spectra were obtained with a Kontron SFM25 spectrofluorimeter at 25 °C. The excitation wavelength used for tryptophan fluorescence was 280 nm. Experiments were carried out in 50 mM Mops/10 % glycerol, pH 7.5.

Stopped-flow spectrofluorimetry

The rate of fluorescence quenching was determined using a Hit-Tech Scientific SF-61-DX2 stopped-flow spectrofluorimeter (Salisbury, U.K.). Fluorescence excitation was at 440 nm using a Xenon lamp, and fluorescence emission was detected above 455 nm with the aid of a cut-off filter. Experiments were carried out in 50 mM Mops/10 % glycerol, pH 7.5, at 25 °C.

CD spectroscopy

CD spectra were recorded with a Jasco J-710 spectropolarimeter at 20 °C with a 2 mm path-length cuvette. A total of 25 spectra were accumulated for each sample and corrected by subtracting contributions from the buffer.

PAGE

Non-denaturing PAGE was performed using the Pharmacia PhastSystem with an 8–25 % gel run at pH 8.8 for 240 A V h over a period of 45 min at 15 °C. The chorismate synthase apoprotein is predicted to have a pI of approx. 6.1 and to have a net charge of between +0.4 and −0.8. Oxidized FMN and substrate were added in 5 µl of between 10 and 50 mM Mops buffer, pH 7.5) as indicated in Figure 4, 20 min before the start of electrophoresis. Jack-bean urease (545 and 272 kDa) and BSA (132 and 66 kDa) were used as protein standards. Denaturing SDS/PAGE was performed as described by Laemmli [15]. Protein standards (Sigma; MW-SDS-70L kit) were: BSA, 66 kDa; egg albumin, 45 kDa; rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine erythrocyte carbonic anhydrase, 29 kDa; bovine pancreas trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; bovine milk α-lactalbumin, 14.1 kDa.

FT-IR spectroscopy

Spectra were recorded with a Perkin–Elmer IR spectrometer (system 2000) equipped with a sample shuttle. The instrument was purged with a continuous flow of dry nitrogen gas both before and during data acquisition. Spectra were calculated from 80 scans obtained at 4 cm⁻¹ resolution over a 20 min acquisition period. Compensation for water vapour was achieved with one scan of the reference cuvette before and after four scans of the sample cuvette. Sample spectra were then corrected with buffer spectra recorded under identical conditions on the same day. Protein concentration was 0.5 mM in 10 mM sodium phosphate buffer, pH 7.5. IR spectra were recorded using calcium fluoride windows using 15 µm spacers at 25 °C.

Small-angle X-ray scattering experiments and data treatment

The synchrotron radiation X-ray scattering data were collected following standard procedures using the European Molecular Biology Laboratory X33 camera [16–18] on the storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY) with multwire proportional chambers and delay line readout [19]. At a sample-detector distance of 2 m and a wavelength λ of 0.15 nm, the range of momentum transfer 0.23 < s < 4.5 nm⁻¹ was covered (s = 4πsinΘ/λ, where 2Θ is the scattering angle). Samples with concentrations of 7–12 mg·ml⁻¹ were used. All data processing procedures (normalization, buffer subtraction, etc.) involved statistical errors propagation using the program SAPOKO (D.I Svergun and M. H. J. Koch, unpublished work).

The maximum diameters of particles were estimated from the scattering curves using the orthogonal expansion program ORTOGNOM [20]. The values of the forward scattering (which is proportional to the molecular mass of the solute) and the radii of gyration were evaluated using the indirect transform program GNOM [21,22].

Shape determination from small-angle X-ray scattering data

The ab initio shape determination from experimental solution scattering curves followed the method of Svergun and Stuhmann [23] and Svergun et al. [24]. The envelope of the particle is represented by an angular border function F(φ), where ω = (Θ, φ) are spherical co-ordinates, which is parameterized as:

\[ F(ω) = \sum_l f_{lm} Y_{lm}(ω) \]  

where \( f_{lm} \) are complex numbers and \( Y_{lm}(ω) \) are spherical harmonics. The resolution of this representation, defined by the truncation parameter \( L \), is equal to \( 4πμR_0/(L + 1) \), where \( R_0 \) is the radius of the equivalent sphere. The algorithms to rapidly calculate the scattering intensity \( I(ω) \) from such a model, taking into account the particle/solvent interface, are described by Svergun [25] and Svergun and Stuhmann [23]. The shape determination was performed using terms up to \( L = 6 \) and assuming a 222-point symmetry of the tetrameric molecule, which yields 13 free parameters \( f_{lm} \) in expansion (see eqn. 1).

These parameters were determined by a non-linear optimization procedure by minimizing the R-factor between the calculated and the experimental curves:

\[ R^2 = \frac{\sum_{j=1}^{N} [(I(s) - I_{exp}(s))/I(s)]^2}{\sum_{j=1}^{N} [I_{exp}(s)/I(s)]^2} \]  

where \( N \) is the number of data points.
where $N$ is the number of experimental points, and $I_{exp}(s)$ and $\sigma(s)$ are the experimental intensity and its S.D. respectively. A sphere was used as an initial approximation; additional penalties to keep the particle surface smooth and the envelope function positive definite are described by Svergun et al. [24]. A constant term was subtracted from each scattering curve prior to the shape determination to remove the unwanted scattering due to the atomicity of the structure and to ensure that the intensity decay was proportional to $s^{-4}$ at higher angles, in accordance with Porod’s law for homogeneous bodies [26].

**Global secondary structure evaluation based on protein CD spectra**

CD spectra were recorded in the range 178–260 nm, and the measured ellipticity was converted into molar CD using the following equation:

$$\text{molar CD} = \frac{(113 \times 30 \times 10^{-6})/(c \times l)}{113 \text{ is the average molecular mass (Da) of an amino acid, } 30 \times 10^{-4} \text{ is the factor used to convert observed ellipticity into } \Delta A, c \text{ is the concentration of the protein in mg ml}^{-1} \text{ and } l \text{ is the path length of the cuvette in cm. The contribution of } \sigma \text{ where } \text{CD}}$$

with Porod’s law for homogeneous bodies [26].

**Secondary and tertiary structure predictions**

The secondary structure of *E. coli* chorismate synthase was predicted using type-1 discrete state-space modelling [28,29]. The 361-amino-acid long *E. coli* chorismate synthase sequence was trimmed by the N-terminal methionine and the last 10 amino acids of the C-terminus, and numbered from 1 to 350 accordingly.

**Determination of strictly conserved amino acids in chorismate synthases**

Strictly conserved amino acids were identified by aligning the following 18 chorismate synthase sequences (accession numbers given in parentheses): *Archaeoglobus fulgidus* (AF0670) [30], *Bacillus subtilis* (P31104), *Corydalis semperirens* (precursor; X60544), *E. coli* (M27714), *Haemophilus influenzae* (P43875), *Helicobacter pylori* (HP0663) [31], *Lycopersicon esculentum* (pre-1) (Z21796), *Lycopersicon esculentum* (precursor 1; Z21791), *Methanococcus jannaschii* (MJ1175) [32], *Mycolobacterium tuberculosis* (P9013), *Neurospora crassa* (U25818), *Plasmidium falciparum* (AF008549), *Saccharomyces cerevisiae* (X60190), *Salmmonella typhimurium* (M27715), *Staphylococcus aureus* (U31979), *Synechymis sp.* strain PCC6803 (P23353), *Toxoplasma gondii* (U93689) and *Vibrio anguillarum* (P39198).

**RESULTS**

**Effects of EPSP and oxidized FMN on the solubility of chorismate synthase**

The solubility of apo-chorismate synthase is approx. 30 $\mu$M in 10 mM potassium phosphate buffer, pH 7.5, at 4 °C. At higher concentrations the protein forms an amorphous white precipitate. The solubility of the enzyme is increased to a small extent with higher phosphate concentrations, the addition of glycerol and an increase in temperature. By contrast, EPSP increases the solubility of the enzyme considerably. The addition of equimolar amounts of EPSP re-solubilizes the precipitated enzyme completely without any detectable loss of the original activity. Protein concentrations of up to approx. 2 mM could be obtained in the presence of EPSP. Oxidized FMN alone does not affect protein solubility.

**Tryptophan fluorescence quenching on binding of oxidized FMN and EPSP**

The tryptophan fluorescence of approx. 90 % (Figure 1). From the graphical data analysis shown in the inset to Figure 1, a dissociation constant of 37 $\mu$M for oxidized FMN was obtained from the slope.
Figure 2  Kinetics of binding of oxidized FMN to chorismate synthase

Oxidized FMN (0.2–1.0 mM after mixing) was rapidly mixed with chorismate synthase (25 µM after mixing) in 50 mM Mops buffer, pH 7.5, using a stopped-flow spectrophuorimeter, and the rate of FMN fluorescence quenching was determined. The \( k_{on} \) and \( k_{off} \) values were determined to be 350 ± 30 M\(^{-1}\) s\(^{-1}\) and 0.02 ± 0.02 s\(^{-1}\) from the slope and intercept respectively of the plot of the observed pseudo-first-order rate against FMN concentration. The value of the correlation coefficient, \( r \), was 0.96, and the bars represent S.E.M. values estimated from triplicate determinations.

Figure 3  Changes in the UV/visible CD spectrum of enzyme-bound oxidized FMN in the presence of EPSP

The observed spectrum of oxidized FMN (66 µM) in 50 mM Mops buffer, pH 7.5, containing 10% glycerol at 20 °C in the presence of chorismate synthase (66 µM) (spectrum 1, dotted line) was corrected for the calculated concentration of enzyme-bound FMN (spectrum 2, dashed line). The spectrum changed upon addition of EPSP (67 µM) (spectrum 3, solid line). Free FMN exhibits little or no CD under these conditions (spectrum 4, solid line).

Rapid kinetics of binding of oxidized FMN to chorismate synthase

When chorismate synthase (25 µM after mixing) was rapidly mixed with an excess of oxidized FMN (0.2–1.0 mM after mixing) in the stopped-flow apparatus, the pseudo-first-order rate of flavin fluorescence quenching showed a linear dependence on the FMN concentration (Figure 2). This experiment yielded values for \( k_{on} \) and \( k_{off} \) of 350 ± 30 M\(^{-1}\) s\(^{-1}\) and 0.02 ± 0.02 s\(^{-1}\) respectively. The large errors, particularly at high FMN concentrations, are a result of the relatively small observable fluorescence change compared with the total fluorescence signal. This is due to the FMN concentration being in excess of the enzyme concentration to allow for pseudo-first-order conditions. A dissociation constant of 60 µM can be calculated from the rate constants. This value is of the same order as, and therefore consistent with, that obtained from the tryptophan fluorescence quenching experiment described above (37 µM).

When chorismate synthase was pre-incubated with EPSP prior to mixing with oxidized FMN, a lower rate of fluorescence quenching was observed. Importantly, the system reached equilibrium within a few minutes in all cases. With a 5–20-fold excess of EPSP over chorismate synthase, this rate was found to be independent of the EPSP concentration. This result suggests that the \( K_d \) for EPSP is lower than that for oxidized FMN, and that there is a compulsory order of binding of oxidized FMN and then EPSP to form a ternary complex where, at high substrate concentrations, the binding of FMN is limited by the dissociation of EPSP from the enzyme. This result is consistent with previous order-of-mixing studies [33], which have shown that the catalytically competent ternary complex of chorismate synthase, reduced FMN and EPSP appears to form with the same order of binding.

Effect of EPSP on the UV/visible CD spectrum of enzyme-bound oxidized FMN

The CD spectrum of oxidized FMN in the presence of chorismate synthase has a negative peak at 445 nm and a positive one at 343 nm (Figure 3, spectrum 1). Since the \( K_d \) of oxidized FMN is 30 µM [9], only 48% of oxidized FMN would have been bound to the enzyme at the concentrations used. The spectrum was adjusted to give that expected for 100% binding (Figure 3, spectrum 2). Addition of an equimolar concentration of EPSP caused a shift of the short-wavelength maximum from 343 to 358 nm and an increase in the resolution of the peak at longer wavelength (Figure 3, spectrum 3). This spectrum required no correction, because the \( K_d \) for oxidized FMN in the presence of EPSP is 20 nM [9], resulting in stoichiometric binding at these concentrations. Free FMN exhibits no strong CD features under these conditions (Figure 3, spectrum 4).

Non-denaturing PAGE of chorismate synthase with and without pre-incubation with EPSP and oxidized FMN

Figure 4 shows a non-denaturing PAGE analysis of chorismate synthase. When applied to the gel alone, more than 90% of the
Figure 5  EPSP and oxidized FMN together protect chorismate synthase from tryptic digestion

Chorismate synthase (14.5 µg), with or without a 3.5-fold molar excess of EPSP and oxidized FMN, was incubated with trypsin (30 ng) for 1 h at 25 °C in 50 mM Mops buffer/10% glycerol, pH 7.5, and subjected to SDS/PAGE with silver staining. Lanes 1, chorismate synthase, no additions; 2, chorismate synthase, EPSP, FMN and trypsin; 3, chorismate synthase, EPSP and trypsin; 4, chorismate synthase, FMN and trypsin; 5, chorismate synthase and trypsin; 6, molecular mass standards indicated in kDa.

Evidence that the binding of EPSP and oxidized FMN protects chorismate synthase from tryptic digestion

Firm evidence that the binding of EPSP and oxidized FMN protects chorismate synthase from tryptic digestion of the protein in the absence and presence of EPSP and oxidized FMN, as shown in Figure 5. In the absence of EPSP and FMN (lane 5), or in the presence of only one of the two (lanes 3 and 4), chorismate synthase was readily digested by trypsin. In the presence of both EPSP and oxidized FMN, however, chorismate synthase was remarkably stable towards the proteolytic activity of trypsin over a period of 1 h at 25 °C (lane 2). The presence of either EPSP alone or FMN alone did not afford even partial protection.

Effects of EPSP and oxidized FMN on the FT-IR spectrum of chorismate synthase

The second derivative spectrum of unliganded chorismate synthase is shown in Figure 7(A). The observed peaks can be assigned to the secondary structure elements as follows [35]: 1691.5 cm⁻¹ (sheets), 1656 cm⁻¹ (β-strands for the amide I region; and 1548.5 cm⁻¹, β-sheet) for the amide II region. Thus the presence of both α-helix and β-sheet is confirmed by this technique. In the ternary complex with EPSP and oxidized FMN (Figure 7B), although the peaks at 1691.5 cm⁻¹ (turns), 1634.5 cm⁻¹ (β-strands), 1549.5 cm⁻¹ (amide II, α-helix) and 1518 cm⁻¹ (amide II, β-strand) remained at similar positions, the signals at 1665 cm⁻¹ (turns) and 1565 cm⁻¹ (α-helix) appeared to be slightly shifted to higher wavenumbers. In addition, new peaks were observed at 1650 cm⁻¹, 1620 cm⁻¹ and 1581 cm⁻¹, as
Figure 7 Changes in the FT-IR spectrum of chorismate synthase on addition of both EPSP and oxidized FMN

Second-derivative spectra are shown of the deconvoluted FT-IR spectra of chorismate synthase (0.5 mM) in the absence (A) and presence (B) of both EPSP and FMN in 10 mM potassium phosphate buffer, pH 7.5, at 25 °C. See the Results section for the peak assignments and a description of the differences between the two spectra.

Well as other minor changes. With free FMN, peaks at 1582 cm⁻¹ and 1549 cm⁻¹ were observed, and free EPSP showed no peak in the spectral range of interest. Therefore the peaks at 1581 cm⁻¹ and the shoulder at 1548 cm⁻¹ can be assigned to the FMN moiety. The two remaining new peaks at 1650 cm⁻¹ and 1620 cm⁻¹ must be attributed to the protein, and are best interpreted as changes in the α-helical and β-strand structural elements of chorismate synthase. Hence formation of the ternary complex of chorismate synthase, EPSP and oxidized FMN appears to generate structural changes in α-helical as well as β-strand secondary-structural elements.

Effects of EPSP and oxidized FMN on small-angle X-ray scattering by chorismate synthase

Solution scattering curves of native chorismate synthase apo-enzyme and the ternary complex with EPSP and FMN are presented in Figure 8. Comparison of the normalized forward scattering with the values obtained with a reference solution of BSA yields a molecular mass of 150 kDa for both forms. This result is in agreement with the estimates from the native gel electrophoresis described above and with earlier reports that chorismate synthase exists as a homotetramer in solution [7]. The maximum size of unliganded chorismate synthase was found to be \( D_{\text{max}} = 11 \pm 0.5 \) nm and its radius of gyration was \( R_g = 3.37 \pm 0.02 \) nm; the parameters of the ternary complex were \( D_{\text{max}} = 10.5 \pm 0.5 \) nm and \( R_g = 3.28 \pm 0.02 \) nm. Furthermore, as seen from the visual comparison of the two experimental data sets in Figure 8, the first subsidiary maximum in the curve from liganded chorismate synthase (indicated by an arrow) is more pronounced than that for unliganded enzyme. This, together with the changes in \( R_g \) and \( D_{\text{max}} \), indicates that the enzyme has a somewhat more compact overall shape upon formation of the ternary complex with EPSP and oxidized FMN.

This qualitative conclusion is further supported by the results of the ab initio shape determination. The particle envelopes restored, as described in the Experimental section at a resolution of 2 nm, are presented in Figure 9, and the fits to the experimental data are shown in Figure 8. The portions of the scattering curves up to \( s_{\text{max}} = 3.5 \) nm⁻¹ were used in the shape determination, and the final R-factors of the restored shapes were 1.0% and 1.1% for unliganded and liganded chorismate synthase respectively. Comparison of the two envelopes indicates that the compaction of liganded chorismate synthase is achieved by ‘correlated screw movements’ of the particle domains which can be identified as monomers. To verify the significance of the observed differences, several shape restorations were performed under different minimization conditions, in particular those where the model of one form was used as an initial approximation to restore the shape of the other; in all cases, shapes very similar to those in Figure 9.
were obtained. The uniqueness of the shape restoration is also supported by the analysis of the information content in the experimental data. As shown by Svergun et al. [21], low-resolution shape determination is unique when the number of independent parameters in the model (M) does not exceed 1.5 times the number of Shannon channels (N_s) in the experimental data, where \( N_s = D_{max}^3/\pi \). In our case, \( N_s = 12 \), and thus the value \( M = 13 \) (see the Experimental section) is well within this limit. Considering the envelopes in Figure 9, one should bear in mind that they were obtained assuming a 222-point symmetry, which allowed a significant decrease in the number of free parameters in the model. Without symmetry, description of a particle envelope up to \( L = 6 \) would require 43 independent parameters. The models should therefore be considered unique only within the symmetry restriction, although assuming a 222 symmetry is justified for a homotetramer. A similar low-resolution model obtained from the solution scattering data for tetrameric yeast pyruvate decarboxylase [36,37] was later confirmed by protein crystallography [38].

**Secondary and tertiary structure prediction based on the amino acid sequence of *E. coli* chorismate synthase**

Since the type-I discrete state-space modelling [28,29] is restricted to a sequence length of up to 350 amino acids, the amino acid sequence of *E. coli* chorismate synthase was trimmed by the N-terminal methionine and the last 10 amino acids of the C-terminus. The C-terminal fragment does not contain any conserved amino acids according to sequence alignments of all known chorismate synthase sequences (see the Experimental section). More importantly, Charles et al. [39] demonstrated that the deletion of the C-terminal 36 amino acids did not affect the correct folding and catalytic activity of the enzyme. In any case, similar modelling results were obtained when the first 11 N-terminal amino acids were omitted.

The sequence analysis shows that *E. coli* chorismate synthase has a probability of 1.0 of belonging to the super-class of \( \alpha \)-\( \beta \)

![Figure 9](image) Changes in the envelopes of chorismate synthase in the presence of both EPSP and oxidized FMN

Low-resolution envelopes of chorismate synthase alone (upper and lower left) are compared with those of the ternary complex of chorismate synthase with EPSP and FMN (upper and lower right) restored from the scattering data (Figure 8). The binding of EPSP and FMN clearly results in correlated screw movements within the homotetrameric enzyme. The lower shapes are 90° anticlockwise rotations along the \( z \)-axis of the upper shapes.

![Figure 10](image) Secondary structure prediction for *E. coli* chorismate synthase and strictly conserved amino acids

The four graphs show the probability of an amino acid residue to be in (from top to bottom) an \( \alpha \)-helix, a \( \beta \)-strand, a loop or a turn, according to type-I discrete state-space modelling. A total of eight repeating \( \alpha/\beta \) units are predicted with high probability, which are consistent with an \( \alpha/\beta \)-barrel. Residues labelled with inverted triangles mark strictly conserved amino acids among the 18 chorismate synthases listed in the Experimental section (17 in \( \alpha \)-helices, 11 in turns, eight in \( \beta \)-strands and seven in loops). Note that the N-terminal methionine and the ten C-terminal amino acids were omitted from the analysis, and the remaining sequence is numbered from 1 to 350 accordingly.
DISCUSSION

In this paper, we present a number of spectroscopic and physical studies which show that chorismate synthase undergoes a major structural change when both oxidized FMN and EPSP are bound. The small-angle X-ray scattering data indicate that the enzyme has a more compact overall shape in the ternary complex. The correlated screw movements are immediately apparent when the shapes are determined from the data. This difference in shape between the two enzyme states is considered to be significant, because very similar models are obtained when the model from one form is used as an initial approximation in the determination of the other. In addition, these experiments show that there is no change in the oligomerization state of the tetrameric enzyme.

The protection from tryptic digestion of the protein in the ternary complex provides additional strong evidence for a structural change. Neither EPSP alone nor FMN alone afforded any such protection. This result is consistent with the decreased reactivity of free cysteine residues with 5,5'-dithiobis-(2-nitrobenzoic acid) on forming the ternary complex [40]. Such a significant structural change could result in changes in the distribution and environment of secondary-structural elements within the protein. A lack of significant changes in the far-UV CD spectrum of the protein indicates that the overall relative proportions of secondary-structural elements do not change significantly. However, changes in the FT-IR spectrum strongly suggest that changes in the environment of both α-helical and β-strand elements do indeed occur.

The ternary complex exhibits increased mobility during non-denaturing PAGE. None of the complex migrated to the same position as the majority of the apoprotein. Since it was subjected to PAGE for a period of 45 min, the complex must be very stable to dissociation, having a half-life at least of the order of tens of minutes. Rather than the increase in mobility being the result of a decrease in hydrodynamic volume, consistent with the small-angle scattering data, it could be due simply to an increase in overall negative charge on binding of the substrate and cofactor. Attempts to address this by using two-dimensional isoelectric focusing PAGE were hampered by precipitation of the apo-enzyme in the gels. Theoretically, the pH of the ternary complex could become more negative by up to 0.5 unit and, at the pH of 8.8 used in the non-denaturing PAGE experiment, the complex could have up to six additional net negative charges. However, the enzyme alone migrated as a diffuse band which extended towards and beyond the position to which the ternary complex migrated. This is difficult to explain in terms of a distribution of differently charged species. It is much more easily explained by a distribution of species with different hydrodynamic volumes, with the majority species having the larger hydrodynamic volume. In other words, these experiments indicate that the apoprotein exhibits a degree of flexibility that the ternary complex does not.

Alterations in the visible CD spectrum of the enzyme-bound FMN on binding of EPSP show that the environment of the flavin changes considerably. Comparisons of spectral alterations with those of other flavoproteins [41–44] for which X-ray crystal structures are known [45,46] indicate that such alterations are not easily rationalized in terms of specific structural changes. In addition, alterations in spectra can occur on binding of substrates adjacent to the flavin, in the absence of any major change in the structure of the protein. However, we cannot rule out the possibility that the alterations observed with chorismate synthase reflect, at least in part, changes in protein structure.

Encouraged by the successful prediction of the tertiary structure of flavodoxin using discrete state-space modelling [28], we have used this method to obtain the first bona fide prediction for the secondary and tertiary structure of chorismate synthase. The results of this method are consistent with those obtained by CD and FT-IR spectroscopy, in that chorismate synthase is composed of both α-helix and β-strands. The percentages of β-strands are in good agreement, but the model predicted a higher α-helical content. The modelling predicted that chorismate synthase has an α-β-barrel fold with a probability of 1.0, and ruled out 15 other possible model structures. Half of all the amino acids predicted to be in turns are strictly conserved among chorismate synthases. Since turns are important structural constraints of a protein structure, this result increases our confidence in the predicted structure. Additional confidence comes from the X-ray crystal structure of the allosteric enzyme pyruvate kinase type I, which shares sequence similarity with the chorismate synthases and has an α-β-barrel at its core [47]. Although the sequence similarity between the two enzymes is significant, it is not sufficient to allow homology modelling.

The bifunctional chorismate synthases from the fungi Neurospora crassa and Saccharomyces cerevisiae have an additional NADPH:FMN oxoreductase activity. The NADPH-binding site has been postulated to involve amino acid residues [48] that map to helix 6, turn 5, strand 6 and turn 6 (see Figure 10). A number of conserved amino acid residues are located in this region. It remains to be seen whether this region forms the structural scaffold for the oxoreductase activity in fungal chorismate synthases. The cluster of conserved amino acids in helix 7 is rich in uncharged small amino acid residues (mainly glycine). By contrast, the cluster in helix 4 includes one acidic and five basic amino acids. It appears likely that this cluster of amino acids in helix 4 could be involved in the binding of the FMN cofactor and of EPSP, and in catalysis. This hypothesis is currently being tested by site-directed mutagenesis experiments using E. coli chorismate synthase.

In summary, we can conclude that chorismate synthase undergoes a major change in protein structure on binding oxidized FMN and EPSP. In addition, electrophoresis experiments indicate that the apoenzyme probably exhibits more conformational flexibility than the ternary complex. The enzyme can bind either oxidized FMN alone, leading to the quenching of tryptophan fluorescence, or EPSP alone, leading to an increase in protein solubility and a delay in FMN binding. However, it is clear that the ternary complex forms only when oxidized FMN binds before EPSP. Furthermore, the increase in the affinity of the enzyme for oxidized FMN and the lowering of the FMN redox potential by EPSP show that their binding sites are not independent [9]. The complete lack of protection by either EPSP alone or FMN alone to tryptic digestion shows that the structural change requires the binding of both. It is not clear whether this tetrameric enzyme exhibits co-operative binding, as no study to date has revealed any. Nevertheless, the stable ternary complex between enzyme, EPSP and oxidized FMN can be considered to be a good structural model of the corresponding active ternary complex formed with reduced FMN immediately before catalysis commences. A similar study using the cofactor and substrate analogues 5-deaza-FMNH₂ [49,50] and (6R)-6-fluoro-EPSP [51], which also preclude normal turnover, would shed more light on the structural change. In the absence of a high-resolution structure of the enzyme, it is not possible to define the nature of the structural changes. However, they are significant changes which are likely to be important during the catalytic cycle of chorismate synthase.

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