STUDIES OF THE STRUCTURE AND CATALYTIC MECHANISM OF CHORISMATE SYNTHASE

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ABBREVIATIONS

The abbreviations used are those recommended in the Biochemical Journal "Instructions to Authors", with the following additions:-

bisTris	bis(2-hydroxyethyl)imino-tris-(hydroxymethyl) methane.
DCPIP	2,6-dichloroindolphenol
DEAE	diethyl aminoethyl
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetate
E_m	emission
E_x	excitation
EPSP	5-enolpyruvylshikimate 3-phosphate
FAD	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
FMN	flavin mononucleotide
$FMNH_2$	reduced flavin mononucleotide
f.p.l.c	fast protein liquid chromatography
h.p.l.c	high pressure liquid chromatography
IPTG	isopropyl-β -D-thiogalactoside
MM+glc	minimal medium + glucose
MTT	thiazolyl blue tetrazolium
NBT	nitroblue tetrazolium
NEM	N-ethyl maleimide
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethyl sulphonyl fluoride
PTH	phenylthionydantoin
SDS PAGE	gel electrophoresis in the presence of sodium dodecyl sulphate
TEA.HCl	trietnanolamine hydrochloride
Ve	elution volume

CONTENTS

LIST OF FIGURES

SUMMARY

1. The established methods for the purification of the N. crassa chorismate synthase have been modified to overcome proteolytic damage and to obtain electrophoretically homogeneous enzyme free from substrates.

2. The purified N. crassa chorismate synthase is a homotetramer with a subunit M_r of 50 000. The enzyme is bifunctional, showing both chorismate synthase and flavin reductase activity.

3. A simple anaerobic assay procedure has been developed which enables chorismate synthase activity to be monitored under strict anaerobic conditions. This procedure was used to detect and purify chorismate synthase from an overproducing strain of E. coli.

4. The purified E. coli chorismate synthase is a homotetramer with a subunit M_r of 38 000. The enzyme is active only in the presence of exogeneously supplied reduced flavin and appears to lack the ability to reduce this cofactor intrinsically.

5. The N-terminal sequence of the E. coli chorismate synthase has been determined for the first thirty residues and matches exactly that predicted from the nucleotide sequence. The complete amino acid sequence of the aroC structural gene is confirmed. The E. coli chorismate synthase shows primary structure homology with internal sequences derived from peptides isolated from the N. crassa enzyme.

6. Gross overexpression of the E. coli chorismate synthase using an IPTG induced overproducing strain resulted in the concomitant overproduction of a yellow chromophore. Spectroscopic and enzymatic analyses indicate that this chromophore is FMN. The E. coli enzyme contains limited amounts of residually bound flavin, as determined by fluorescence spectroscopy. Both the N. crassa and E. coli chorismate syntnase have a preference for FMN over FAD as the flavin cofactor. A putative FMN-phosphate binding region is identified from the E. coli chorismate synthase amino acid sequence.

7. Analysis of aromatic and flavin fluorescence spectra indicate that in vivo the E. coli chorismate synthase subunit is the binding site for FMN. The N. crassa enzyme is highly sensitive to thiol directed agents and insensitive to metal activation or metal chelation.

8. All of the structural and kinetic analyses presented suggest that the N. crassa enzyme is a fusion protein; a chorismate synthase domain coupled to a flavin reductase activity.

9. Preliminary evidence suggests that neither a thiol nor a metal is involved at the chorismate synthase active site, but that flavin itself could adequately assume the role of a nucleophile in a two-step reaction mechanism.

CHAPTER 1 INTRODUCTION TO CHORISMATE SYNTHASE

1.1 Introduction

1.1.1 The shikimate pathway

Plants and microorganisms synthesise the three aromatic amino acids tyrosine, tryptophan and phenylalanine as well as many other biologically important molecules from the carboxylic acid chorismate. (Figure 1.1). This branchpoint intermediate is in turn synthesised via the shikimate pathway, its carbon skeleton derived from two products of carbohydrate metabolism, erythrose-4-phosphate and phosphoenolpyruvate (Figure 1.2).

Davis (1955) and Sprinson (1960) elucidated the steps and intermediates of the shikimate pathway by studying auxotrophic mutants of Escherichia coli. The pathway to chorismic acid is common to bacteria, fungi and plants but is not found in other eukaryotic organisms. The shikimate pathway and the synthesis of aromatic compounds has since been extensively reviewed (Haslam, 1974; Weiss & Edwards, 1980; Conn, 1986).

1.1.2 The organisation of the genes and enzymes of the shikimate pathway

The organisation of the genes and enzymes of the shikimate pathway in a number of different organisms (prokaryotic and eukaryotic) is found to differ markedly. In the bacteria E. coli, Salmonella typhimurium and Bacillus subtilis the genes encoding the shikimate pathway enzymes, steps 2-6, are generally widely scattered around the chromosome (Bachman, 1983; Sanderson & Roth, 1983; Henner & Hoch, 1980). In contrast these genes are found to be clustered in a variety of lower eukaryotic organisms; Neurospora

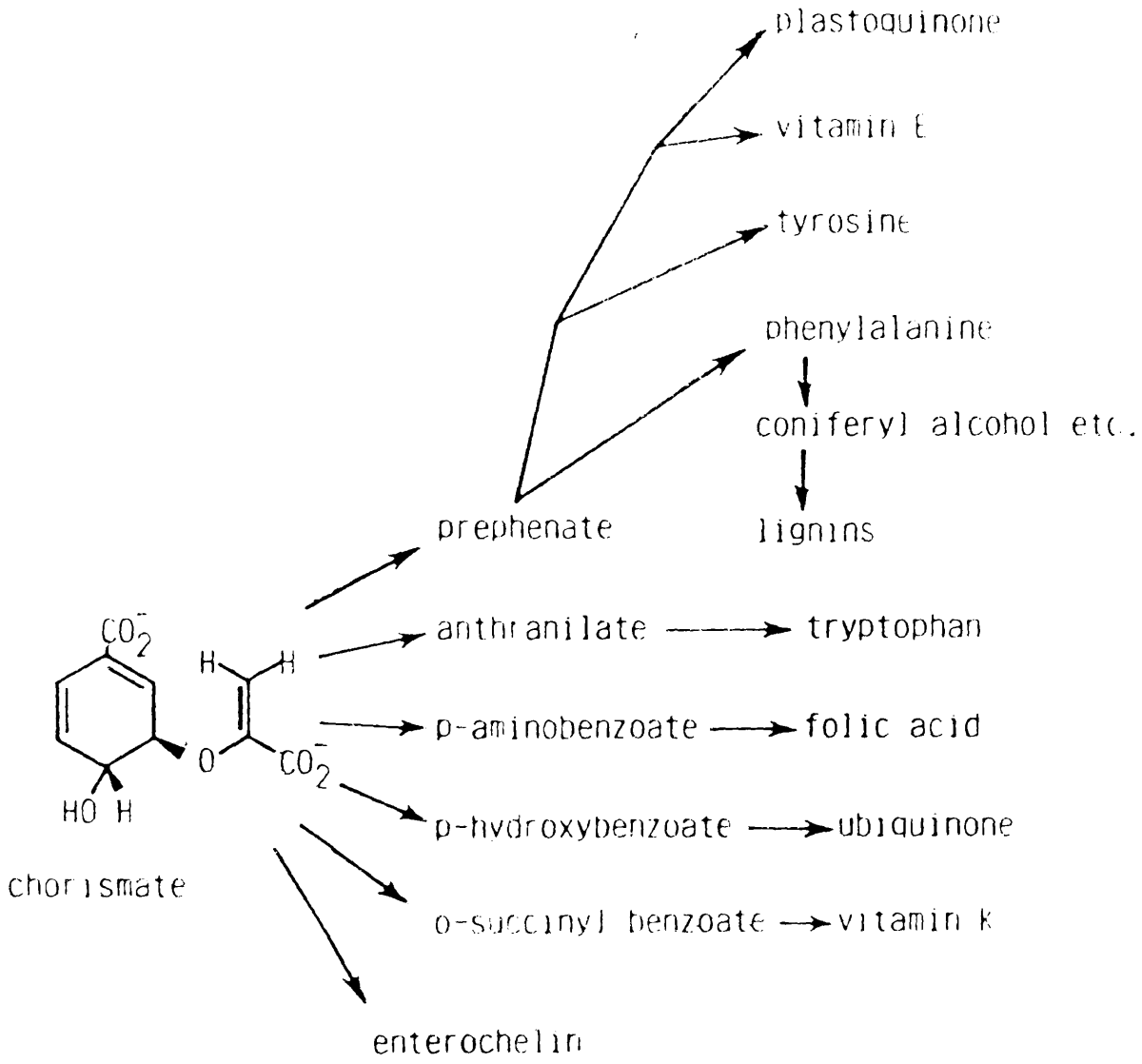


Figure 1.1 The utilisation of chorismate

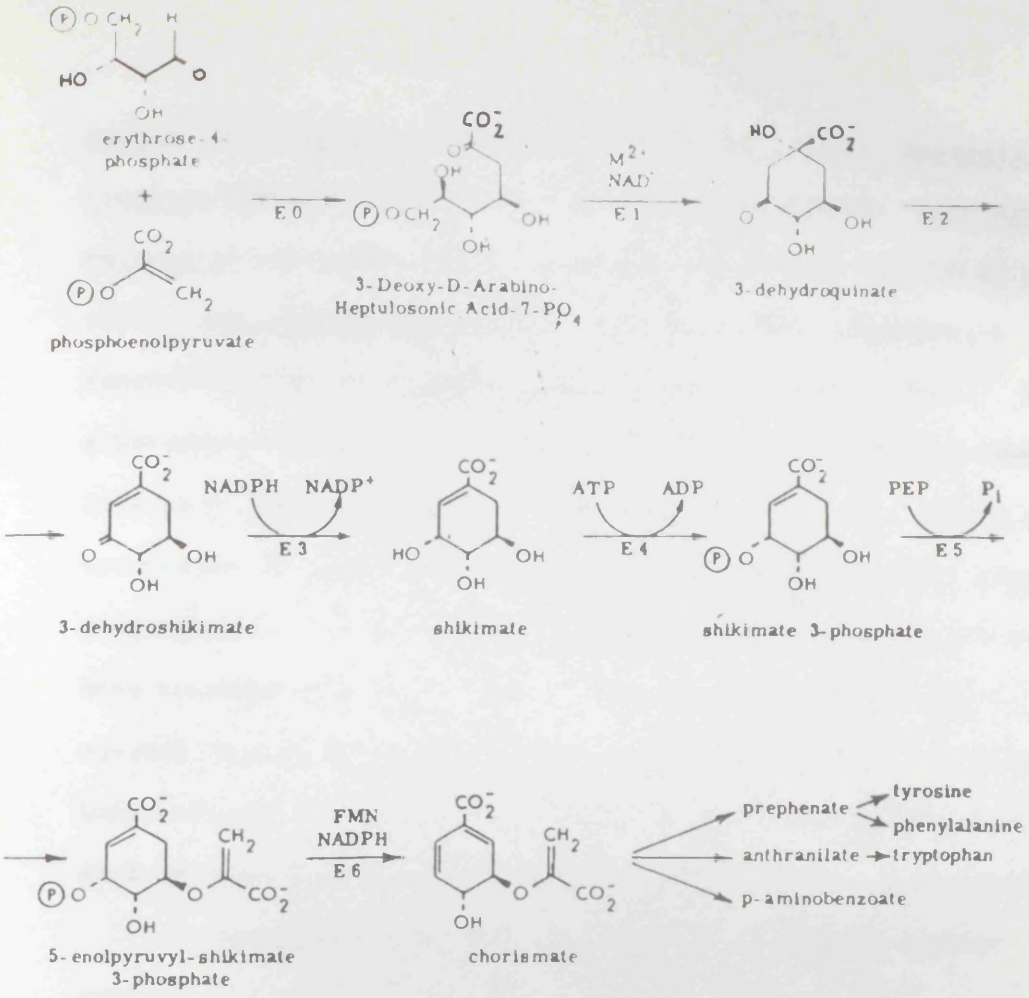


Figure 1.2 The shikimate pathway

Abbreviations used:

E0	DAHP synthase	EC 4.1.2.15
E1	DHQ synthase	EC 4.6.1.3
E2	dehydroquinase	EC 4.2.1.10
E3	shikimate dehydrogenase	EC 1.1.1.25
E4	shikimate kinase	EC 2.7.1.71
E5	EPSP synthase	EC 2.5.1.19
E6	chorismate synthase	EC 4.6.1.4
DAHP	3-deoxy-D-arabino-heptulosonate-7-phosphate	
DHQ	3-dehydroquinate	
DHS	3-dehydroshikimate	
Shik3P	shikimate-3-phosphate	
EPSP	5-enolpyruvylshikimate-3-phosphate	

crassa (Giles et al., 1967; Catchside et al., 1985), Aspergillus nidulans (Ahmed & Giles, 1969; Charles et al., 1986), Saccharomyces cerevisiae (de Leeuw, 1967; Larimer et al., 1983, Duncan et al., 1987), Schizosaccharomyces pombe (Strauss, 1979; Nakanishi & Yamamoto, 1984) and Euglena gracilis (Berlyn et al., 1970). It has subsequently been shown that the shikimate pathway enzymes (steps 2-6) in S. cerevisiae and A. nidulans are encoded on a single gene, the product of which is a pentafunctional polypeptide (the arom polypeptide). Within these polypeptides exist five domains which show homology with each of the corresponding monofunctional E. coli enzymes (Duncan et al., 1987; Charles et al., 1986). It appears that the arom polypeptide is a mosaic of five functional domains, each of which is homologous to a monofunctional E. coli polypeptide.

The structural organisation of the shikimate pathway enzymes in a number of organisms is quite heterogeneous. Fungi and yeast appear to have five of the seven reactions linked on a single polypeptide chain. In E. coli each of the seven pathway enzymes have been shown to be separable (Chaudhuri & Coggins, 1985; Coggins et al., 1985). In plants three of the enzymes are separable but two, 3-dehydroquinase and shikimate dehydrogenase, co-purify and have been shown to occur as a bifunctional polypeptide (Coggins, 1986; Mousdale et al., 1987). In the fungus N. crassa the arom multifunctional enzyme has been purified to homogeneity and shown to catalyse steps 2-6 in the pathway (Lumsden & Coggins, 1977; Boocock, 1983; Lambert et al, 1985; Coggins & Boocock, 1987). The bacterium B. subtilis highlights a more complex situation. In this organism there appears to exist both multifunctional polypeptides and multienzyme complexes. The enzymes dehydroquinate

synthase and chorismate synthase exist as a trifunctional enzyme complex along with a flavin reductase (diaphorase) activity (Hasan & Nester, 1978c). A bifunctional polypeptide has also been identified, catalysing the first reaction of the shikimate pathway [3-deoxy-D-arabino-hephtulosonate phosphate (DAHP) synthase] and a later reaction in aromatic biosynthesis (chorismate mutase). This polypeptide was found in non-covalent association with another shikimate pathway enzyme, shikimate kinase (Huang et al., 1973; Huang et al., 1975).

1.1.3 The enzymes and reactions of the shikimate pathway

The enzymes and reactions of the shikimate pathway have been the subject of intensive study since their elucidation in the early 1950's. Some of the intermediates pose interesting synthetic problems and the chemistry and enzymology of the pathway is still poorly understood (Ganem, 1978). Recently the realisation that the pathway could be used as a target for 'ecologically sound' herbicides and antimicrobial agents nas focused renewed attention on a number of the shikimate pathway enzymes (Coggins, 1986; Amrhein, 1986).

1.2 Chorismate synthase

Chorismate synthase catalyses the seventh and final reaction in shikimate pathway. Chorismic acid is generated from EPSP by the release of one mole of H_3PO_4 (Figure 1.3). All chorismate synthases studied to date show activity only in the presence of a reduced flavin cofactor, either $FMNH_2$ or $FADH_2$, which is not stoichiomet rically consumed during the reaction (Morell et al., 1967; Welch et al., 1974; Hasan & Nester, 1978;

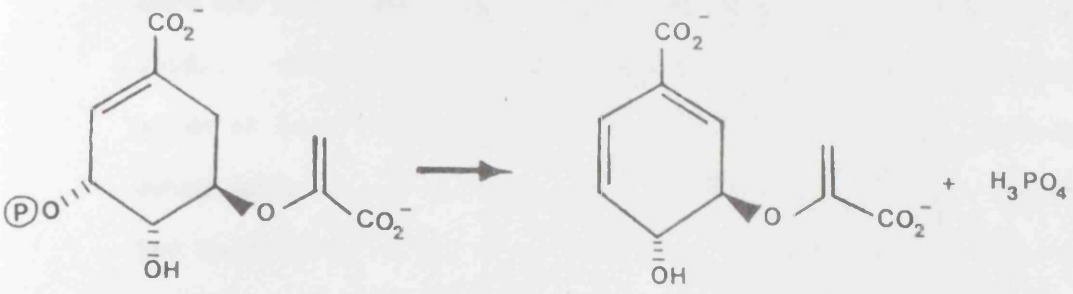


Figure 1.3' The reaction catalysed by chorismate synthase

Boocock, 1983; Mousdale & Coggins, 1986). This requirement is unusual as the overall reaction does not involve any net change in redox state.

1.2.1 The reaction mechanism of chorismate synthase

The stereospecificity of the enzymic synthesis of chorismic acid has been demonstrated (Hill & Newkome, 1969; Onderka & Floss, 1969). Hill & Newkome used stereospecifically deuterated shikimic acids as carbon sources for a mutant strain of E. coli lacking dehydroquinase and shikimate dehydrogenase activities. Analysis of the endproducts tyrosine and phenylalanine showed that only the 6-R hydrogen was lost (Figure 1.4), indicating that the overall transformation was a trans-1,4-elimination reaction. This result is at odds with the stereoselectivity predicted when taking into account orbital symmetry expectations where a simple concerted E2 process is expected to proceed by a cis-elimination of the two leaving groups (Floss et al., 1972; Ganem, 1978). In an attempt to explain the anomalous anti-stereoselectivity Onderka & Floss proposed a two step ($S_N2/E2$) mechanism for the action of chorismate synthase. Firstly a nucleophilic group on the enzyme attacks the carbon-carbon double bond in the EPSP ring eliminating phosphate (H_2PO_3). An enzyme-bound intermediate is formed. There is then an internal rearrangement involving the return of electrons to the nucleophile and the elimination of the 6-R hydrogen to form chorismate (Figure 1.5a). An alternative E1 type mechanism could also generate the correct stereochemistry. This two step reaction would involve the formation of a carbonium ion via the elimination of phosphate. Deprotonation, perhaps with the aid of a base on the enzyme, would yield chorismate with the correct

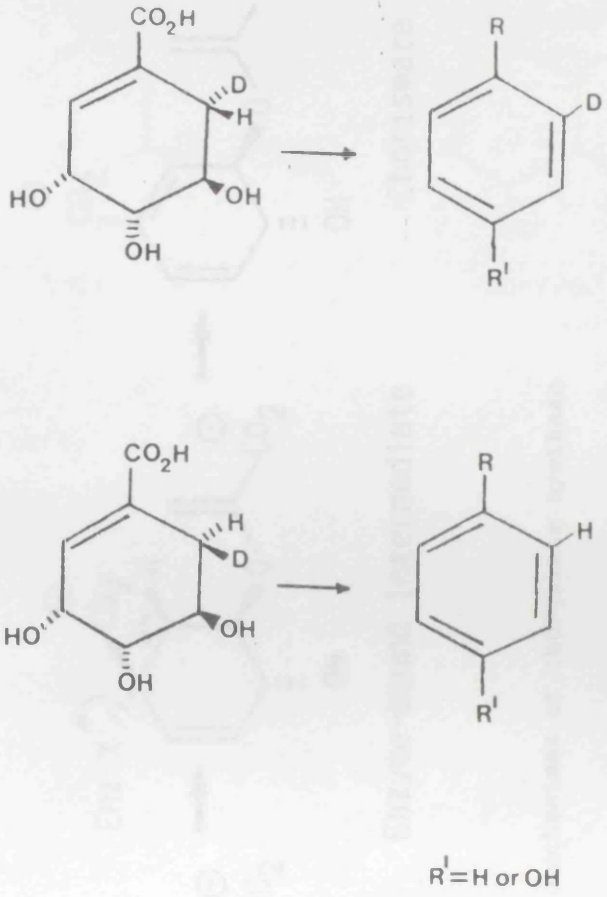


Figure 1.4 The stereospecificity of chorismate synthase

A TWO STEP MECHANISM FOR CHORISMATE SYNTHASE

(Onderka & Floss, 1972)

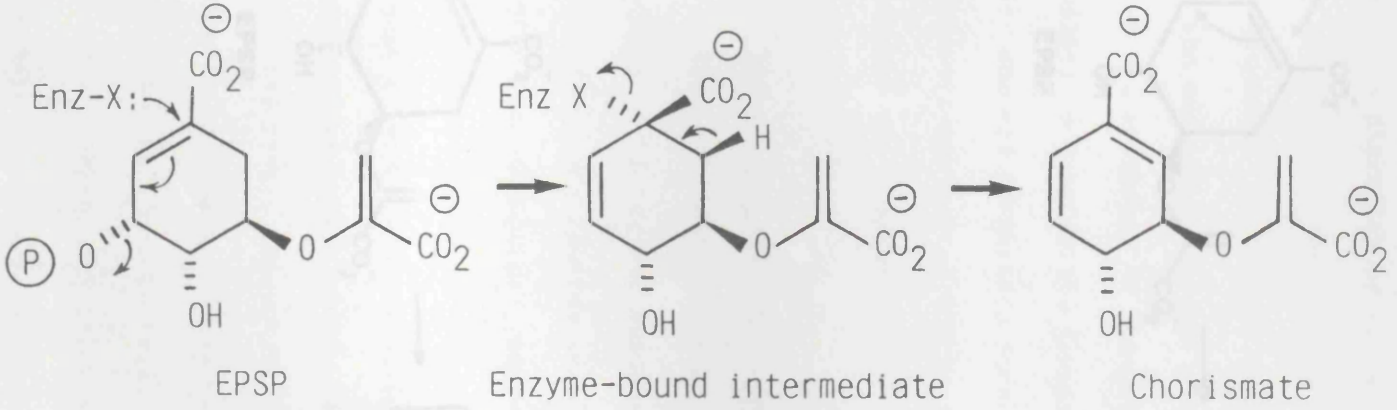
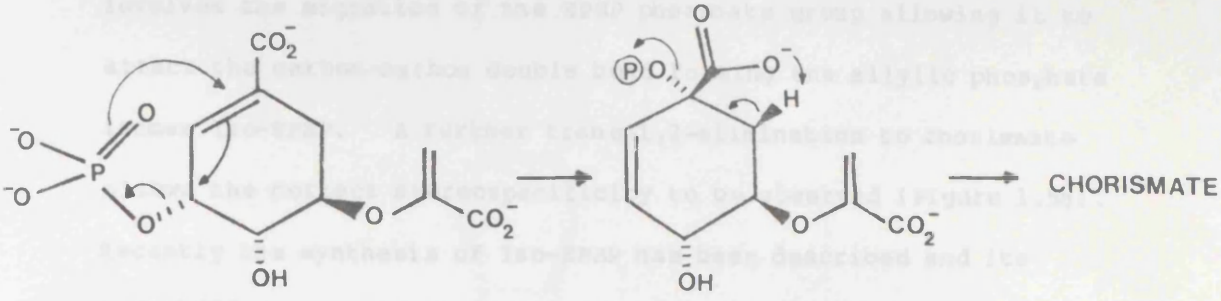
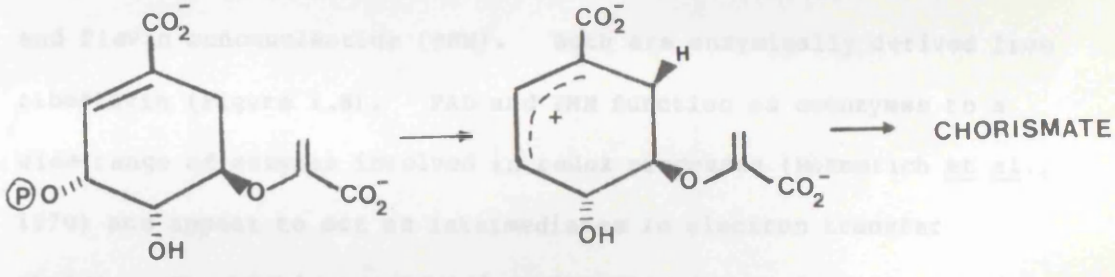


Figure 1.5 Possible reaction mechanisms of chorismate synthase

Figure 1.5 (cont'd).



B

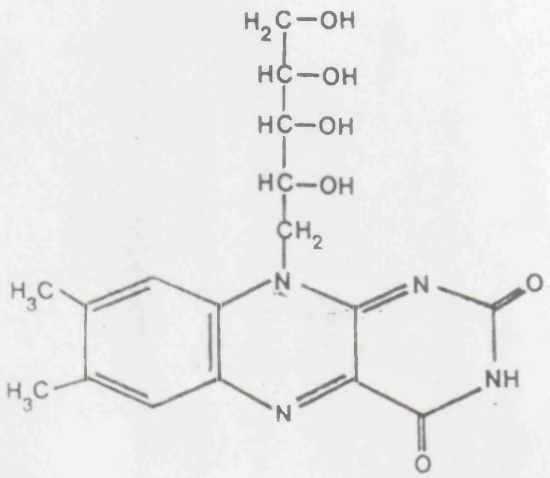


C

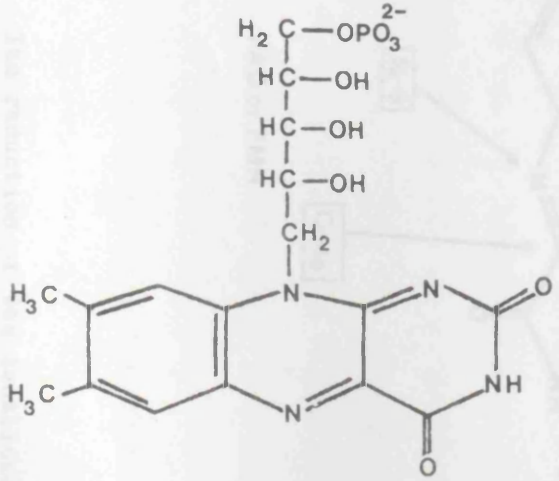
stereoselectivity. (Figure 1.5c). Finally, Ganem (1978) suggested that the reaction mechanism could proceed *via* an allylic rearrangement followed by an *anti*-E2 elimination. This reaction involves the migration of the EPSP phosphate group allowing it to attack the carbon-carbon double bond forming the allylic phosphate isomer Iso-EPSP. A further trans-1,2-elimination to chorismate allows the correct stereospecificity to be observed (Figure 1.5b). Recently the synthesis of Iso-EPSP has been described and its suitability as a substrate investigated (Bartlett *et al.*, 1986). Bartlett and his co-workers found that although Iso-EPSP was a good competitive inhibitor of the *N. crassa* chorismate synthase the compound was not a substrate.

1.3 The role of flavin in enzyme catalysis

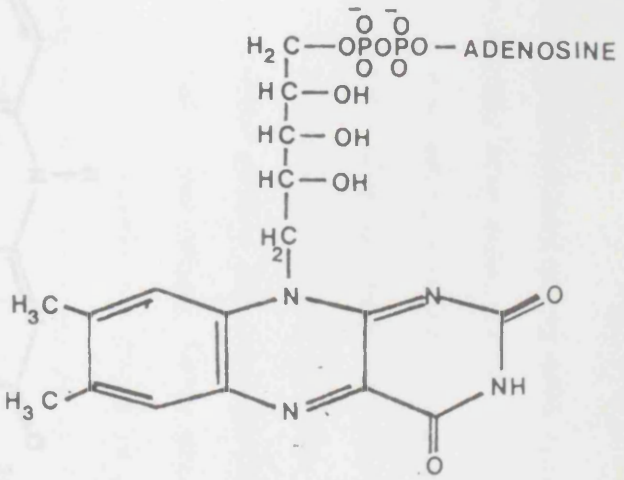
Flavins are derivitives of isoalloxazine and fulfill a unique role in biological oxidation-reduction processes. In nature the two most common flavins are flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Both are enzymically derived from riboflavin (Figure 1.6). FAD and FMN function as coenzymes to a wide range of enzymes involved in redox processes (Hemmerich *et al.*, 1970) and appear to act as intermediates in electron transfer chains. The highly conjugated, tricyclic ring system allows these molecules to be ideally suited to act as 'electron sinks'. The oxidised forms can undergo readily reversible two-electron reduction chemically or enzymically (Figure 1.7). Reduction is accompanied by a characteristic loss of absorbance at 450nm. Flavins can also undergo single-electron transfers to produce half-reduced radical forms (semiquinones). These species (like the fully reduced



RIBOFLAVIN



FMN



FAD

Figure 1.6 The structures of riboflavin, FMN and FAD

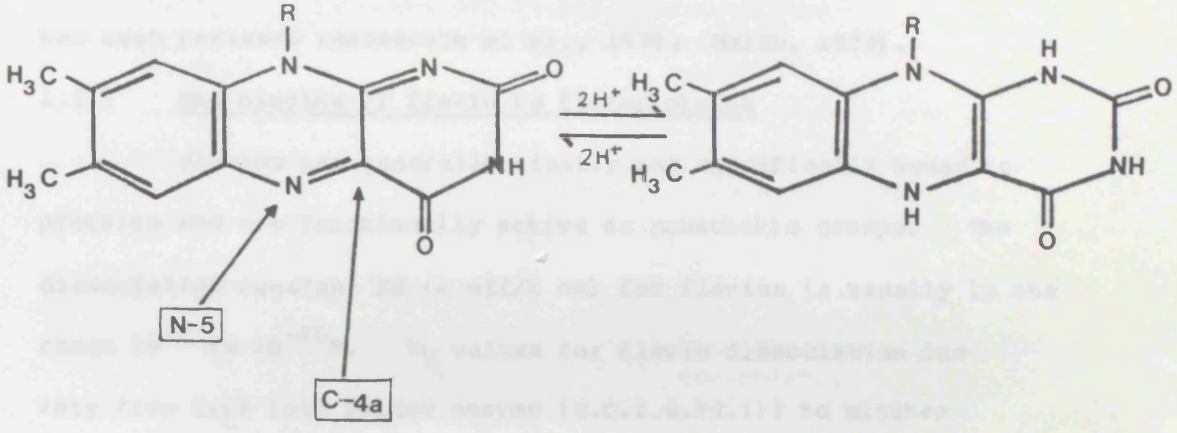


Figure 1.7 The reduction of the isoalloxazine ring system

flavins) are unstable in aqueous solution, but are often stabilised and prevented from reoxidising by complexing with protein.

The reactive centres of the oxidised or reduced isoalloxazine ring system are the C-4a and N-5 atoms. The N-5 position is involved in the transfer of hydride equivalents from reduced nicotinamides and both centres are involved in covalent addition reactions (Walsh, 1979; Bruice, 1976). Although the biochemistry of flavin catalysis is poorly understood the subject has been reviewed (Hemmerich et al., 1970; Walsh, 1979).

1.3.1 The binding of flavin to flavoproteins

Flavins are generally tightly and specifically bound to proteins and are functionally active as prosthetic groups. The dissociation constant Kd (k off/k on) for flavins is usually in the range 10^{-7} to 10^{-10}M. $T_{1/2}$ values for flavin dissociation can vary from days [old yellow enzyme (E.C.1.6.99.1)] to minutes [D-amino acid oxidase (Dixon & Kleppe, 1965)]. Flavin can also be covalently bound and some 25 different enzymes from bacteria to the highest vertebrates, have been shown to contain covalently linked flavin (Singer & McIntyre, 1984). More recently it has been shown that the ability of a protein to bind and stabilise its flavin prosthetic group can be influenced by the redox state of that group. Or more simply some flavoproteins show a greater affinity for reduced flavin over oxidised flavin and vice versa (Madden et al., 1984).

1.3.2 Flavoprotein classification

One convenient way of classifying flavoproteins is according to the nature of the final electron acceptor (Walsh, 1979). Table 1.1 outlines four categories of flavoproteins. The

Table 1.1

Classification of flavoenzymes by electron acceptors

	Category	Acceptors
1.	Dehydrogenases	Quinones, cytochromes or iron-sulphur clusters. Not O_2.
2.	Oxidases	O_2
3.	Flavodoxins	Electron transport chain components.
4.	Redox inactive	-

dehydrogenases do not use oxygen as an acceptor and must prevent autoxidation of the bound and reduced flavin group. This may be achieved by either exclusion of oxygen from the active site or by orientating the flavin moiety in such a way as to prevent oxidation. The oxidases react readily with oxygen, and this reaction is often enhanced by the involvement of a divalent metal ion. The flavodoxins are a very homologous group of FMN containing flavoproteins and are among the strongest of biological reductants. The final category is comprised of those flavoproteins that catalyse reactions involving no net redox change (redox inactive flavoproteins). To date four such enzymes have been identified, studied and assigned to this group; mandelonitrile lyase (oxynitrilase) EC.4.1.2.10) (Jörns, 1979; Xu et al., 1986), tartronate semialdehyde synthase (glyoxalate carboligase) (E.C.4.1.1.47) (Cromartie & Walsh, 1976), acetolactate synthase (E.C.4.1.3.18) (Schloss et al., 1985) and chorismate synthase (E.C. 4.4.1.4.) Morell et al., 1967; Welch et al., 1974; Hasan & Nester, 1978b; Boocock, 1983; Mousdale & Coggins, 1986; White et al., 1987). The glyoxalate carboligase and acetolactate synthase enzymes require tightly bound FAD, a divalent metal ion and thiamine pyrophosphate (TPP) for catalytic activity, but there is no evidence that the flavin moiety is involved in any redox type mechanisms. The oxynitrilase enzymes show a requirement for bound FAD, but again there is no evidence for any redox reaction involving the flavin group. It has been proposed (Jörns, 1979) that these enzymes have evolved from a precursor flavoenzyme which may have lost the ability to catalyse redox reactions, while retaining the coenzyme as an integral structural component necessary for enzymic activity.

Chorismate synthase is unique among these redox inactive flavoproteins in that reduced flavin is the cofactor necessary for the enzyme to be catalytically active, and in vivo reducing equivalents must be enzymically generated. It is therefore implied that the flavin group may play a much more significant role in enzyme catalysis than a mere structural component. The role of flavin in the reaction mechanism of this enzyme will be discussed in the next section and in Chapters 7 and 8.

1.4 The role of flavin in the synthesis of chorismate

The role of reduced flavin in the reaction mechanism of chorsimate synthase is not mechanistically obvious. Several hypotheses have been postulated;

1. Reduced flavin is required to reduce an oxygen sensitive iron atom at the active site of the enzyme. [Fe(III) ⟶ Fe(II)] (Morell et al., 1967).

2. Reduced flavin reduces a disulphide bridge to an active site thiol (Welch et al., 1974) which could then function as a catalytic nucleophile in the two-step reaction mechanism proposed by Onderk & Floss (1972).

3. Reduced flavin is an active nucleophile in the reaction mechanism (Boocock, 1983).

4. Reduced flavin has only a structural role, analogous to the flavin moieties of the other redox inactive flavoproteins described in the previous section.

1.4.1 How flavin is reduced by chorismate synthase

Chorismate synthase has been characterised from three microbial sources [E. coli (Morell et al., 1967), N. crassa (Welch et al., 1974; Boocock, 1983) and B. subtilis (Hasan & Nester, 1978b)]. Although all the enzymes studied show a requirement for reduced flavin their ability to generate this cofactor differs markedly. Both the N. crassa and B. subtilis chorismate syntnases appear to be associated with specific flavin reductase (diaphorase) activites that can generate the reduced flavin via the oxidation of nicotinamide nucleotides under aerobic conditions. In contrast, the partially purified E. coli and pea enzyme could only be assayed under strictly anaerobic conditions in the presence of chemically or enzymically reduced flavin (Morell et al., 1967; Mousdale & Coggins, 1986; White et al., 1987). The in vivo source of reducing equivalents for these enzymes is not immediately obvious.

1.4.2 The structural organisation of chorismate synthases

Prior to this study only two chorismate synthases had been purified to apparent homogeneity, the enzymes from the fungus N. crassa and the bacterium B. subtilis (Welch et al., 1974; Boocock, 1983; Hasan & Nester, 1978).

1.4.2.1 N. crassa chorismate synthase

The N. crassa chorsimate synthase has been shown to have a subunit M_r of between 50 and 55000. Chorismate synthase activity appears to copurify with a specific flavin reductase activity.

1.4.2.2 B. subtilis chorismate synthase

In the bacterium B. subtilis chorismate synthase is found as a trifunctional enzyme complex with another of the shikimate pathway enzymes (dehydroquinate synthase) and a specific flavin

reductase activity. This complex dissociates under denaturing conditions to yield three polypetides of M_r 24000 (chorismate synthase), 17000 (denydroquinate synthase) and 13000 (flavin reductase).

1.5 Aims of this project

Chorismate synthase is an unusual enzyme which catalyses an unusual reaction mechanism. The enzyme has been studied in detail from only two organisms and snows much catalytic and structural heterogeneity. The aims of this project are two-fold.

1. To compare the bacterial (*E. coli*) and fungal (*N. crassa*) enzyme and to investigate their ability to generate reduced flavin.

2. To try to understand the reaction mechanism of chorismate synthase and in particular the role of the reduced flavin cofactor.

The first step is the purification to homogeneity of chorismate syntnase from both organisms. This will make it possible to undertake a detailed structural and kinetic comparison of the two enzymes.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and biochemicals

Tris, triethanolamine HCl (TEA HCl) and NADPH (Tetrasodium salt) were obtained from Boehringer Corp., Lewes, East Sussex.

FMN (sodium salt), FAD (Disodium salt), riboflavin, phenylmethylsulphonyl fluoride (PMSF), Bis-tris and Coomassie Brilliant Blue G250 were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

DTT (dithiothreitol), KH_2PO_4, K_2HPO_4, urea, ammonium bicarbonate, disodium EDTA and all reagents for gel electrophoresis were obtained from BDH Chemicals, Poole, Dorset, U.K.

The barium salt of 5-enolpyruvylshikimate 3-phosphate, prepared as described in Coggins et al. (1987), was a gift from Mrs. S. Muir. Dimethyl suberimidate dihydrochloride was prepared as described by Davies & Stark (1970) and was a gift from Professor J.R. Coggins.

Benzamidine HCl and N-ethylmaleimide (NEM) were from Aldrich Chemical Co., Gillingham, Dorset, U.K. All other chemicals were of ANALAR grade or of the highest grade available.

2.1.2 Chromatography media

DEAE-Sephacel, phenyl-Sepharose CL-4B and Sephacryl S300 (superfine) were obtained from Pharmacia, Milton Keyes, Bucks., U.K. Phosphocellulose (P11) and DEAE-cellulose (DE52) were from Whatman Biochemicals, Maidstone, Kent, U.K.

2.1.2.1 F.P.L.C.

F.P.L.C. was performed using Mono Q (HR5/5) and Superose 6 (10/30) prepacked columns in a standard Pharmacia f.p.l.c. apparatus.

2.1.3 Enzymes and proteins

The following enzymes were obtained from Boehringer Corp.:

aldolase (EC 4.1.2.1.3) from rabbit muscle

carbonic anhydrase (EC 4.2.1.1.) from bovine erythrocytes

catalase (EC 1.11.1.6) from beef liver

deoxyribonuclease I (EC 3.1.4.5) from bovine pancreas

β-galactosidase (EC 3.2.1.23) from E. coli

glutamate dehydrogenase (EC 1.4.1.3) from beef liver

hexokinase (EC 2.7.1.1.) from yeast

lactate dehydrogenase (EC 1.1.1.27) from pig muscle

malate dehydrogenase (EC 1.1.1.37) from pig heart

pyruvate kinase (EC 2.7.1.40) from rabbit muscle

The following proteins were obtained from Sigma Chemicals Co.;

myoglobin

bovine serum albumin

cytochrome c from horse heart

phosphorylase a (EC 2.4.1.1) from rabbit muscle

Horse apoferritin was obtained from Mann Research Laboratory, Division of Becton, Dickinson.

Clostripain (EC 3.4.22.8) was obtained from the Worthington Biochemical Corportation, Freehold, New Jersey, U.S.A.

Fatty acid synthetase was a gift from Dr. H.G. Nimmo, Department of Biochemistry, University of Glasgow.

2.1.4 N.crassa and E. coli strains

N.crassa strain 74-OR23-1A (F.G.S.C. No. 987) was obtained from the Fungal Genetics Stock Centre, Humboldt State University

Foundation, Arcata, CA 95521, U.S.A.) was grown harvested and stored as described by Lumsden & Coggins (1977). The cells were grown by J. Greene & S. Muir.

E.coli strains and plasmids;

strain	genotype	source/reference
E. coli K12	wild type ATCC 14948, F^-, lysogenic, Lederberg strain W3100	American Type Culture Collection (Rockville, Maryland U.S.A)
E. coli AB2849	aroC$^-$ derivitive of E. coli K12	CGSC:- (E. coli Genetic stock centre, Department of Human Genetics, Yale University, New Haven, U.S.A.) Pittard & Wallace (1966).
E. coli AB2849/pGMo02		Dr. G. Millar, Department of Biochemistry, University of Glasgow (Millar et al, 1986)
E. coli AB2849/pGMo05		Dr. G. Millar, Department of Biochemistry, University of Glasgow.

2.2 General methods

2.2.1 pH measurements

pH measurements were made with a Radiometer pH probe, calibrated at room temperature.

2.2.2 Conductivity

Conductivity measurements were made at 4°C with a Radiometer conductivity meter type CDM2e (Radiometer, Copenhagen, Denmark).

2.2.3 Protein estimation

Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard.

2.3 Polyacrylamide gel electrophoresis

Electrophoresis was performed by the method of Davis (1964) in 5% polyacrylamide gels at 4°C. After electrophoresis gels were stained either for protein or for diaphorase activity, Electrophoresis in the presence of SDS was performed by the method of Laemmli (1970), with a 3% stacking gel and a 10% running gel, or by the method of Weber & Osborn (1969) in 5% tube gels. The ratio of acrylamide: bis-acrylamide in all PAGE experiments was 30:0.8. Polymerisation was induced by 0.03% (v/v) N,N,N',N' -tetramethylethylene diamine and 0.05% ammonium persulphate.

2.3.1 Staining

(a) Protein staining

Unless otherwise stated gels were stained for protein with 0.1% coomassie brilliant blue G250 in 10% v/v glacial acetic acid, 50% v/v methanol at 40°C and destained at room temperature in 10% acetic acid, 10% methanol. Gels were stained for protein by the silver method as described by Wray et al. (1981).

(b) Activity staining

Non-denaturing tube gels were stained for diaphorase activity at 21°C in a cocktail consisting of 0.25mM-NADPH, 100uM-FMN, 60uM-2,6-dichloroindo phenol and 1mM-thiazolyl blue tetrazolium in 100mM-Tris/HCl buffer, pH 8.8.

2.4 Preparation and standardisation of EPSP

EPSP was prepared enzymically from PEP and shikimate 3-phosphate, and was isolated as the barium salt essentially as described by Knowles et al. (1970). The barium salt was added to 5mM-K_2SO_4 to give a 1mM stock solution of the potassium form of EPSP, which was then standardised by enzymic conversion to chorismate ($\varepsilon_{275} = 263 M^{-1} cm^{-1}$; Gibson, 1970).

2.5 Enzyme assays

Spectrophotometric assays were performed at 25°C in a total volume of 1.0ml. The instrument used was a Gilford-Unicam model 252 spectrophotometer equiped with a Gilford photoelectric detector and recorder. Separation and detection of chorismate in stopped time assay cocktails was performed using a 'fast organic acids; cation exchange h.p.l.c column. A Gilson 303 pump and a Gilson Holochrome variable wavelength U.V. detector were used for h.p.l.c separation. The U.V. absorbance was monitored at 275nm,

2.5.1 N.crassa chorismate synthase

N.crassa chorismate synthase was assayed routinely by monitoring the formation of chorismate spectrophotometrically at 275nm in a continuous assay. The assay cocktail contained (final concentrations) 50mM-triethanolamine hydrochloride/KOH buffer, pH 7.0, 50mM-KCl, 200uM-NADPH, 10uM-FMN and 50uM-EPSP.

2.5.2 E. coli chorismate synthase

E. coli chorismate synthase was assayed routinely under anaerobic conditions by measuring the appearance of chorismate in a stopped time method (White et al., 1987) (see Chapter 4). The version of the assay described in Chapter 4 is essentially the same as in White et al. (1987) but with the following improvements; enzyme sample is added to initiate the reaction and the h.p.l.c. solvent is 40mM-H_2SO_4.

2.5.3 N.crassa chorismate synthase anaerobic assays

N.crassa chorismate synthase was assayed anaerobically following the procedure developed for the assay of the E. coli enzyme (Chapter 4). The standard 1ml assay contained 50mM-potassium phosphate buffer pH 7.0, 50uM-EPSP, 10uM-FMN and 2mM-sodium dithionite. The assay was initiated by the addition of enzyme sample.

2.5.4 The diaphorase activity of chorismate synthase

Diaphorase activity was assayed spectrophotometrically by following the reduction of the electron acceptor cytochrome c. The assay mixture contained 50mM-triethanolamine hydrochloride/KOH buffer, pH 7.0, 50mM-KCl, 50uM NADPH, 10uM FMN and 0.5mg/ml cytochrome c. All assays were conducted at 548nm (ε max of the band of the reduced cytochrome) and assuming ε_{548} = 20,000.

2.6 Growth of E. coli strains

2.6.1 Media

Minimal medium + glucose (MM+glc) consisted of M9 salts plus 0.2% (w/v) glucose and 10uM $CaCl_2$.

M9 salts contained (per litre):

$Na_2 HPO_4$ 6g

$KH_2 PO_4$ 3g

NH_4Cl 1g

$MgSO_4 7H_2O$ 0.13g

Nutrient broth medium was 13g oxoid nutrient broth per litre.

L-Broth medium contained (per litre):

bacto -tryptone 10g

yeast extract 5g

NaCl 10g

and 0.1% (w/v) glucose.

All media were autoclaved at 5 p.s.i for 50 minutes.

2.6.2 Growth of E. coli K12

E. coli K12 cells were grown by J. Greene and S. Muir. All cells were grown at 37°C.

Nutrient broth (50ml) was inoculated with a loop of stock culture and grown overnight. One ml of this culture was then used to inoculate 50ml MM+glc,. this culture was then grown overnight. Eight flasks containing 50ml MM+glc were each inoculated with 1ml of this overnight culture, and grown for 8 hours. Large scale growth of E. coli K12 was in 3 litres MM+glc in 10 litre flasks, each 3 litres of MM+glc was inoculated with 2x50ml MM+glc

cultures. These 3 litre cultures were stirred constantly and compressed air introduced into each flask at 400ml/min. Growth was monitored at 420nm and the cells harvested when the A_{420} was between 3.5 and 5.0 (late logarithmic phase). The cells were harvested by centrifugation at 6000g for 15 mins at 4°C. The cell pellets were resuspended and washed with 100mM-Tris/HCl pH 7.5.

The washed cells were then pooled and recentrifuged prior to storage at -20°C. A yield of 25-30g of E. coli K12 cells (wet weight) could be expected to be obtained from 12 litres of MM+glc.

2.6.3 Growth of E. coli AB2849/pGM602

A stock culture of E. coli AB2849/pGM602 in L-Broth containing 40% (v/v) glycerol was a gift from Dr. Gary Millar.

L-Broth (10ml) containing 100ug/ml ampicillin, was inoculated with E.coli AB2849/pGM602 and grown overnight. One ml of this culture was then added to each of four 50ml culture flasks containing MM+glc+ampicillin (100ug/ml). These cultures were grown overnnight and then the whole contents used to inoculate 4x36 MM+glc+ Ampicillin (100ug/ml) in 10 litre flasks. Large cultures were stirred constantly and aerated with compressed air (400ml/min). The cells were grown to late log phase before harvesting, washing and storage as a paste at -20°C. Twelve litres of medium usually gave a yield of 20-30g of cells (wet weight). Cells were grown by J. Greene and S. Muir.

2.6.4 Growth of E. Coli AB2849/pGM605

A stock culture of E. coli AB2849/pGM605 in L-Broth containing 40% (v/v) glycerol was a gift from Dr. Gary Millar.

E. coli AB2849/pGM605 cells were grown on L-Broth suplemented with ampicillin (100ug/ml). An overnight culture of

cells (120ml) was used to inoculate 8x500ml cultures of MM+glc+Ampicillin (100ug/ml) in 2 litre shaking flasks. After 1 hour incubation, inducer (IPTG) was added to a final concentration of $5x10^{-4}$M and incubation continued for a further 9 hours. The cells were then harvested and washed as described previously. Cells were stored as a paste at -20°C. 10-15g of cells (wet weight) was a typical yield using this procedure.

2.6.5 E. coli cell breakage

E. coli cells for enzyme purification were resuspended in ice cold extraction buffer and broken by two passages through an automatic French pressure cell at 98MPa (14 300 p.s.i internal pressure). The cell was pre-cooled on ice before use (Cat. no. 4-3398A, American Instruments Company, Maryland, U.S.A.).

2.7 Protein sequencing

2.7.1 Acid-washed glassware

Glassware for protein chemistry was washed overnight in concentrated nitric acid, then rinsed exhaustively with glass distilled water. The glassware was then dried in a hot oven.

2.7.2 Carboxymethylation

Prior to sequencing protein samples were reduced and carboxymethylated by the method of Lumsden & Coggins (1978). Samples were dialysed exhaustively against 0.5% (w/v) ammonium bicarbonate and then lyophilised. The lyophilised protein was resuspended in 2ml 0.1M Tris-HCl pH 8.2, 8M Urea (recrystallised), 2mM DTT and incubated in the dark for 1 hour (room temperature) under N_2. This solution was then made 15mM in iodoacetate and incubated for 1 hour. The reaction was terminated by the addition

of DTT to a concentration of 30mM. The carboxymethylated protein was then exhaustively dialysed against 0.5% (w/v) ammonium bicaronate and lyophilised.

2.7.3 Preparation of peptides from the N.crassa chorismate synthase

A 6nmol sample of the N.crassa chorismate synthase which had been reduced and carboxymethylated was digested with clostripain by following the procedure of Mitchell & Harrington (1971). Clostropain was first activated by pre-incubation in 1mM-calcium acetate/2mM-DTT overnight at 4°C. The digestion was carried out in 75mM-sodium phosphate buffer pH7.6, 7.5mM-DTT at a final protein concentration of 5mg/ml. The previously activated clostripain was added to give a protein/protease ratio of 50:1 (w/w). Digestion was allowed to proceed for 1 hour at 21°C and for a furtner 3 hours at 37°C. The reaction was terminated by freezing at -20°C. The resulting peptides were separated by reverse-phase chromatography on a Waters u Bondapak C_{18} column (see Chapter 5).

2.7.4 Gas-phase sequencing

The peptides isolated from the N.crassa chorismate synthase were sequenced on an Applied Biosystems model 4708 gas phase sequencer as described by Russel et al. (1986). The instrument was operated by Mr. B. Dunbar of Aberdeen University. The phenylthiohydantoin samples were analysed by chromatography on a Waters Resolve C_{18} reverse phase column with a pH 5.0 acetate/acetonitrile buffer system (Carter et al., 1983).

2.7.5 Liquid-phase sequencing

A 30 nmol sample of the E. coli chorismate synthase which had been reduced and carboxymethylated was sequenced on a Beckman

model 890 liquid-phase sequencer, as described previously (Smith et al., 1982), operated by Mr. B. Dunbar of Aberdeen University. The phenylthiohydantoin samples were analysed as described in the previous section. The automatic protein sequencing described in the previous two sections was carried out on the SERC funded protein sequencing facility at Aberdeen University with assistance from Mr. B. Dunbar and Professor J.E. Fothergill.

2.8 Molecular weight determinations

Standard molecular weight proteins were selected from the list of proteins and enzymes given in section 2.1.3.

2.8.1 Subunit M_r

SDS PAGE was used to estimate the subunit M_r of the purified proteins. The proteins used to produce standard curves of R_f vs log M_r are listed below

Protein	Subunit M_r
1. phosphorylase a	94 000
2. bovine serum albumin	66 000
3. catalase	60 000
4. glutamate dehydrogenase	56 000
5. aldolase	40 000
6. lactate dehydrogenase	36 000
7. carbonic anhydrase	29 000

Values for subunit molecular weights were taken from Weber & Osborn (1969).

2.8.2 Native M_r

Conventional and Superose 6 gel permeation chromatography were used to estimate the native M_r of the purified proteins.

(a) Sephacryl S-300

A column (60cmx1.1cm) of Sephacryl S-300 was equilibrated in 50mM-Tris-HCl pH 7.5, 50mM-KCl, 0.4mM-DTT at 4°C. The flow rate was 3ml/h and 1.0ml fractions were collected. The column void volume was measured using blue dextran. The elution volumes (V_e) of a number of proteins of known M_r were determined and a standard curve of peak elution vol (ml) *vs* molecular weight constructed. The standard proteins used to calibrate the column were: 1, myoglobin (M_r 17 200); 2, carbonic annydrase (M_r 29 000); 3, malate dehydrogenase (M_r 70 000); 4, lactate dehydrogenase (M_r 144 000); 5, pyruvate kinase (M_r 237 000); 6, apoferritin (M_r 440 000).

(b) FPLC gel-permeation

The native M_r values of both enzymes were estimated by gel-permeation chromatography at room temperature on a Superose 6 column in a Pharmacia f.p.l.c. apparatus. The column was eluted with 50mM-Tris-HCl pH 7.5, 150mM-KCl and 0.4mM-DTT (flow rate 0.5ml/min, fraction size 0.25ml). The eluate was monitored at 280nm and the column was calibrated with the following proteins: 1, yeast hexokinase (M_r 104 000); 2, pig muscle lactate dehydrogenase (M_r 144 000); 3, rabbit muscle pyruvate kinase (M_r 237 000); 4, horse apoferritin (M_r 440 000).

2.9 Crosslinking with dimethylsuberimidate

This was carried out in 0.1M-triethanolamine buffer pH 8.0 as described previously (Lumsden & Coggins, 1977) except that the final concentrations were 0.3mg of protein/ml 20mM-dimethylsuberimidate and 40mM NaCl. After 1 hour the

crosslinking reaction was terminated by the addition of 1M-NH_4HCO_3 to a final concentration of 0.5M. After dialysis into 0.05M-sodium phosphate pH 7.2 the crosslinked proteins were then analysed by electrophoresis in 0.05M-sodium phosphate buffer, pH 7.2, in the presence of SDS (Weber & Osborn 1969).

2.10 Fluorometric analyses

Fluorometric analyses were performed at room temperature using a Perkin Elmer LS Luminescence Spectrometer attached to a Perkin Elmer R100A recorder. Excitation and emission slit widths were set at 10nm and unless stated otherwise final sample volumes were 2ml Flavin excitation spectra were obtained by measuring the emission at 540nm and exciting with wavelengths between 300 and 510nm. Flavin emission spectra were obtained by exciting at a wavelength of 450nm and measuring the emission of light between 480 and 600nm. Tryptophan emission spectra were obtained by exciting at 280nm and measuring the emission between 300 and 400nm.

2.11 Absorption spectra

Absorption spectra were determined at room temperature using a Cecil Instruments CE 505 Double Beam U.V. Spectrophotometer. Final sample and reference volumes were 1ml.

CHAPTER 3 THE PURIFICATION AND PRELIMINARY CHARACTERISATION OF THE N. CRASSA CHORISMATE SYNTHASE

3.1 Introduction

This chapter describes the development of an improved purification procedure for the preparation of electrophoretically homogeneous chorismate synthase from N. crassa. Some structural properties of the purified enzyme are also described.

3.1.1 The need for an improved purification scheme

Previous attempts to purify chorismate synthase (Gaertner & Cole, 1973; Welch et al., 1974) pre-dated the recognition of the protease problem in N. crassa (Lampkin et al., 1976; Gaertner & Cole, 1976; Lumsden & Coggins, 1977) and the realisation of the potential of cellulose phosphate as a pseudo affinity matrix for this and other phosphate binding proteins (Cole & Gaertner, 1975). Yields of the purified enzyme were low (< 5%) and the lack of a stringent anti-protease strategy raised doubts as to the structural integrity of the polypeptide chain. The use of phosphocellulose chromatography aided in the development of a new purification procedure (Boocock, 1983); a final substrate elution step from cellulose phosphate yielded homogeneous enzyme. Although this material was electrophoretically pure the procedure used prohibitive quantities of expensive substrate (EPSP) and gave low yields of enzyme that could not be guaranteed free of substrates. To permit a detailed structural and kinetic analysis of this enzyme a purification scheme was developed to obtain structurally intact, electrophoretically homogeneous chorismate synthase of high specific activity free from potentially interfering substrates. This procedure utilised both phosphocellulose and high resolution

chromatography in conjunction with a full anti-protease strategy similar to that employed in the purification of the arom multifunctional enzyme from the same organism (Lumsden & Coggins, 1977).

3.2. Purification Procedure

Unless otherwise stated all steps after the breaking of the cells were performed at $4^{o}C$.

Step 1: Extraction and centrifugation.

A 90g batch of N.crassa powdered, freeze-dried mycelia was stirred gently into 1500ml of an extraction buffer which consisted of 100mM-potassium phosphate pH7.0. This buffer was supplemented with 5mM-EDTA, 0.4mM-DTT and 1.2mM-PMSF. Stirring was continued for 1h. The extract was then centrifuged at 10 000g for 30min. The supernatant was passed through several layers of cheesecloth and was the crude extract from which the enzyme was purified.

Step 2: "Negative" chromatography on DEAE-cellulose.

The crude extract was pumped at 400ml/h through a column (15cm x $61cm^{2}$) of DE52 pre-equilibrated with 100mM-potassium phosphate buffer, pH7.0 containing 0.4mM-DTT and 1.2mM-PMSF. The column was washed with equilibration buffer until all significant chorismate syntnase activity had eluted.

Step 3: Fractionation with $(NH_4)_2SO_4$

The material eluted from the DE52 column was made 1mM with respect to benzamidine and adjusted to 40% saturation with solid $(NH_4)_2SO_4$ (242g/l). After the mixture had been stirred for 20min the precipitated proteins were removed by centrifugation for 30min at 10 000g. The supernatant was adjusted to 50% saturation with solid $(NH_4)_2SO_4$ (63g/l) and stirred for 20min. The precipitated protein was collected by centrifugation for 30min at 10

000g and redissolved in 100ml of 100mM-potassium phosphate buffer pH7.0 containing 0.4mM-DTT and 1.2mM-PMSF. The dissolved protein was then dialysed overnight against 2x1 litres of 50mM-Tris/HCl buffer, pH7.5, containing 0.4mM-DTT and 1.2mM-PMSF (buffer A).

Step 4: DEAE-Sephacel chromatography.

The dialysed protein was spun briefly at 10,000g to remove any precipitated proteins and then applied to a DEAE-Sephacel column ($12cmx5cm^2$) that had been pre-equilibrated with buffer A. The column was then washed with buffer A plus 30mM-KCl (flow rate 200ml/h) until the A_{280} was below 0.15. The protein was eluted with a 1 litre linear gradient of KCl (30-300mM) in buffer A (flow rate 100ml/h; 10ml fractions). The fractions containing highest enzyme activity were pooled and dialysed against 2x2 litres of 10mM-potassium phosphate buffer, pH6.5, containing 0.4mM-DTT and 1.2mM-PMSF (buffer B).

Step 5: Phosphocellulose chromatography.

The dialysed protein from the previous step was applied to a phosphocellulose column ($1cm^2x25cm$) that had been pre-equilibrated with buffer B. The protein was eluted with two 200ml linear gradients of potassium phosphate in buffer B:- 10-100mM(flow rate 30ml/h; 10ml fractions) and 100-400mM (flow rate 12ml/h; 5ml fractions). Chorismate synthase activity eluted in the second gradient (Figure 3.1) and fractions of high activity were pooled and dialysed overnight against 1 litre of buffer A. The protein was concentrated by adsorption on a 1ml (bed volume) DEAE-Sephacel column and eluted with buffer A containing 1M-KCl. The concentrated enzyme was dialysed against 1 litre of 20mM-Bis-Tris/HCl pH6.0 containing 0.4mM-DTT and 1.2mM-PMSF (buffer C) prior to chromatography on Mono Q.

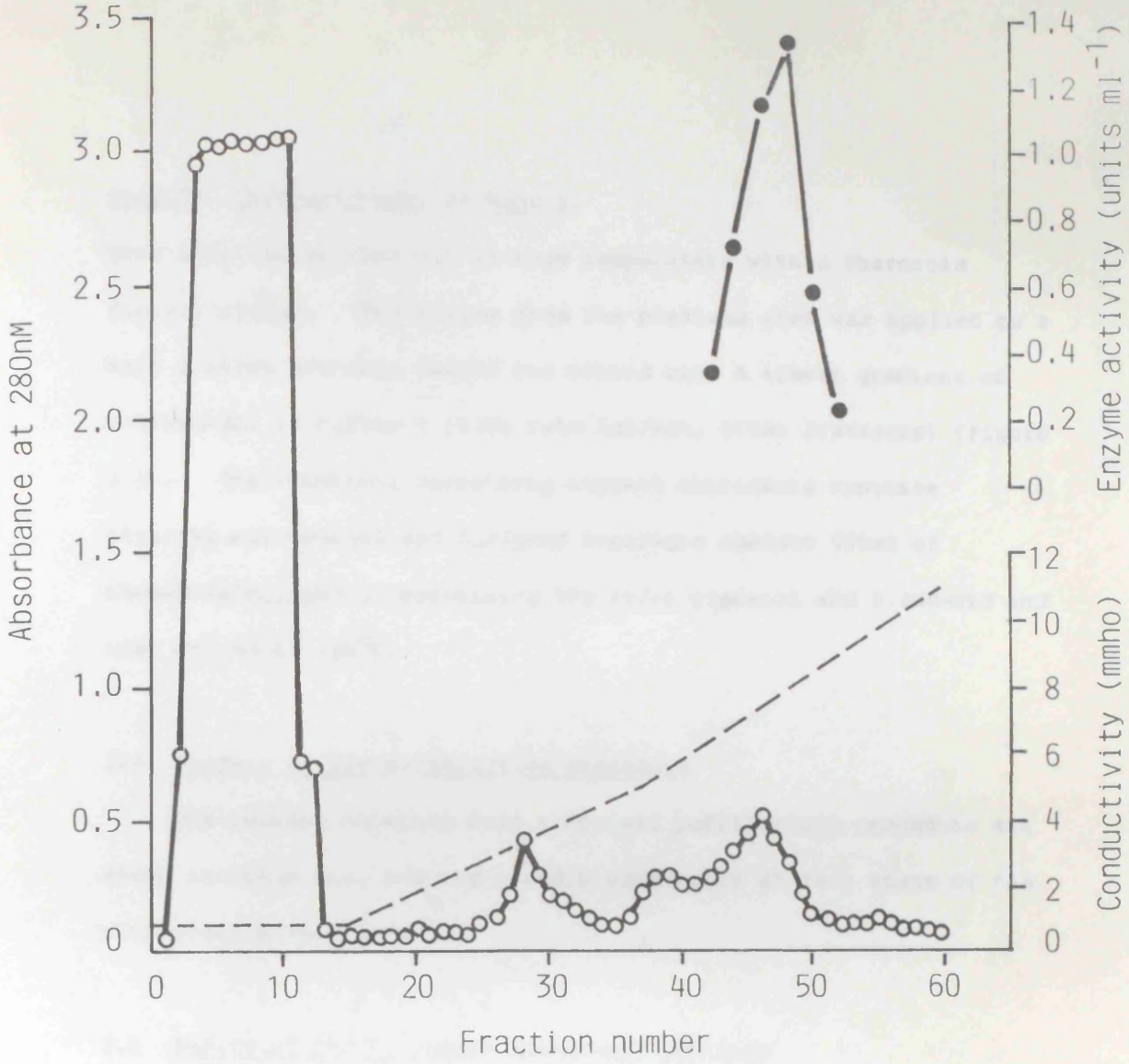


Figure 3.1 Chromatography on phosphocellulose

Enzyme from chromatography on DEAE-Sephacel was loaded on to a column of phosphocellulose and eluted with a phosphate gradient, as described in Section 3.2.
(o), A_{280}; (•), chorismate synthase activity; (--), conductivity.

Step 6: Chromatography on Mono Q.

This step was carried out at room temperature with a Pharmacia f.p.l.c system. The enzyme from the previous step was applied to a Mono Q anion exchange column and eluted with a linear gradient of 0-300mM-KCl in buffer C (flow rate 1ml/min, 0.5ml fractions) (Figure 3.2). The fractions containing highest chorismate synthase activity were pooled and dialysed overnight against 500ml of 50mM-Tris/HCl pH7.5, containing 50% (v/v) glycerol and 0.4mM-DTT and then stored at -20^{o}C.

3.3 Summary of the purification procedure

The results obtained from a typical purification procedure are shown in Table 3.1, and the protein components at each stage of the purification in Figure 3.3.

3.4 Purity of the N. crassa chorismate synthase

The N. crassa chorismate synthase was judged to be electrophoretically homogenous under non denaturing and denaturing conditions. Non denaturing PAGE of the purified enzyme showed a single band on staining for protein. This corresponded with a single band of diaphorase activity (Figure 3.4). SDS PAGE of the purified enzyme also showed a single band when stained for protein (Figure 3.3, track F).

3.5 Molecular weight

A structural analysis of the N. crassa chorismate synthase was undertaken to determine the quaternary structure of the enzyme. Gel filtration experiments utilising both conventional and high

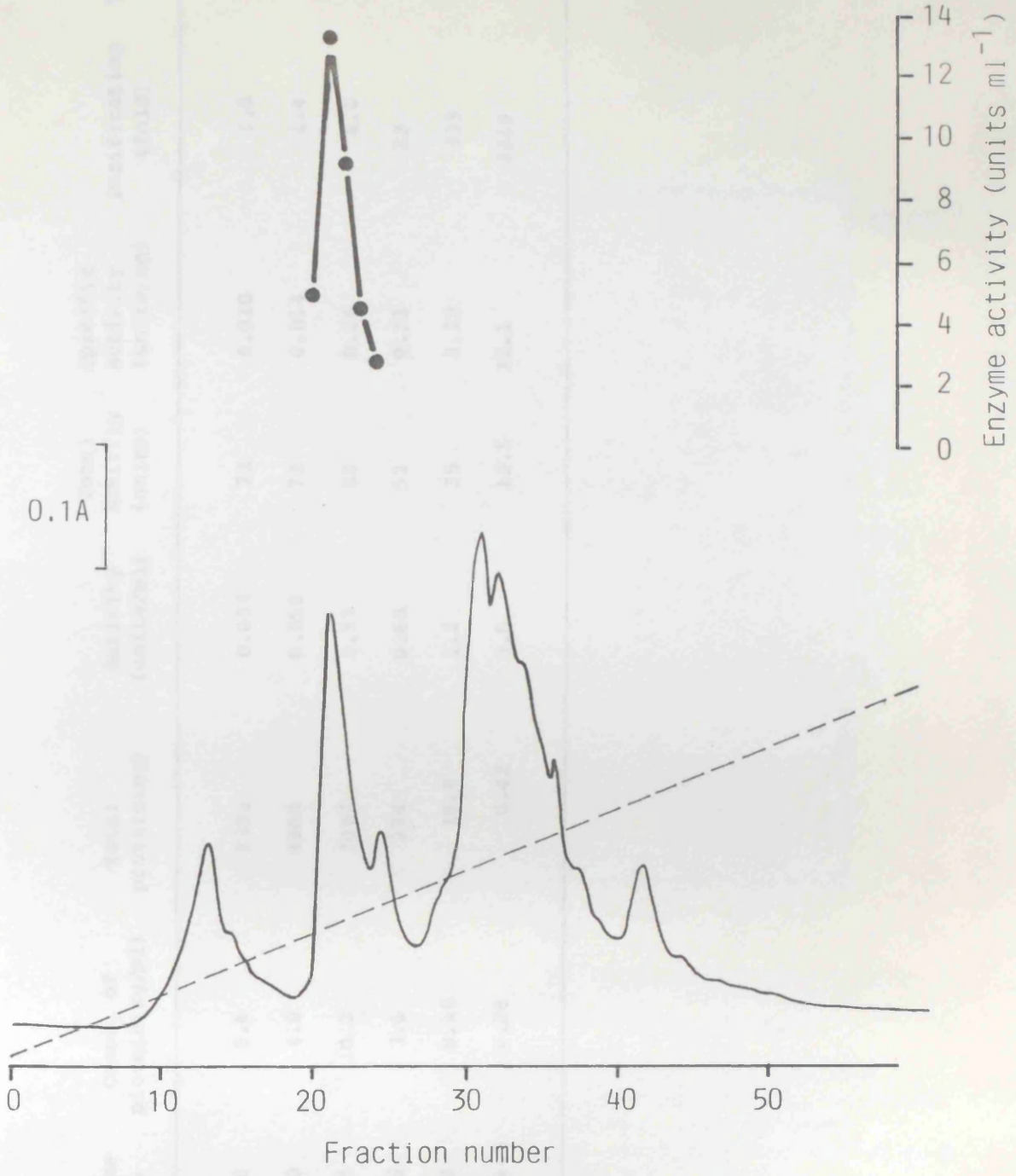


Figure 3.2 Chromatography on Mono Q

Enzyme from chromatography on phosphocellulose was loaded on to a Mono Q column and eluted with a salt gradient, as described in Section 3.2.
(—), A_{280}; (•) chorismate synthase activity; (--), salt gradient.

Table 3.1 Purification scheme for N. crassa chorismate synthase

The results represent a purification from 90g freeze-dried powdered mycelium.

	Step	Volume (ml)	Concn. of protein(mg/ml)	Total protein(mg)	Activity (units/ml)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
1:	Crude extract	1320	5.6	7392	0.054	72	0.010	1.0	100
2:	-ve DE 52	1240	4.0	4960	0.058	72	0.014	1.4	100
3:	40-50% $(NH_4)_2SO_4$	105	10.3	1082	0.57	60	0.06	6.0	83
4:	DEAE-Sephacel	86	2.6	224	0.60	52	0.23	23	72
5:	Cellulose Phosphate	30	0.36	10.8	1.2	36	3.33	333	50
6:	Mono Q	1.5	0.28	0.42	9.0	13.5	32.1	3210	19

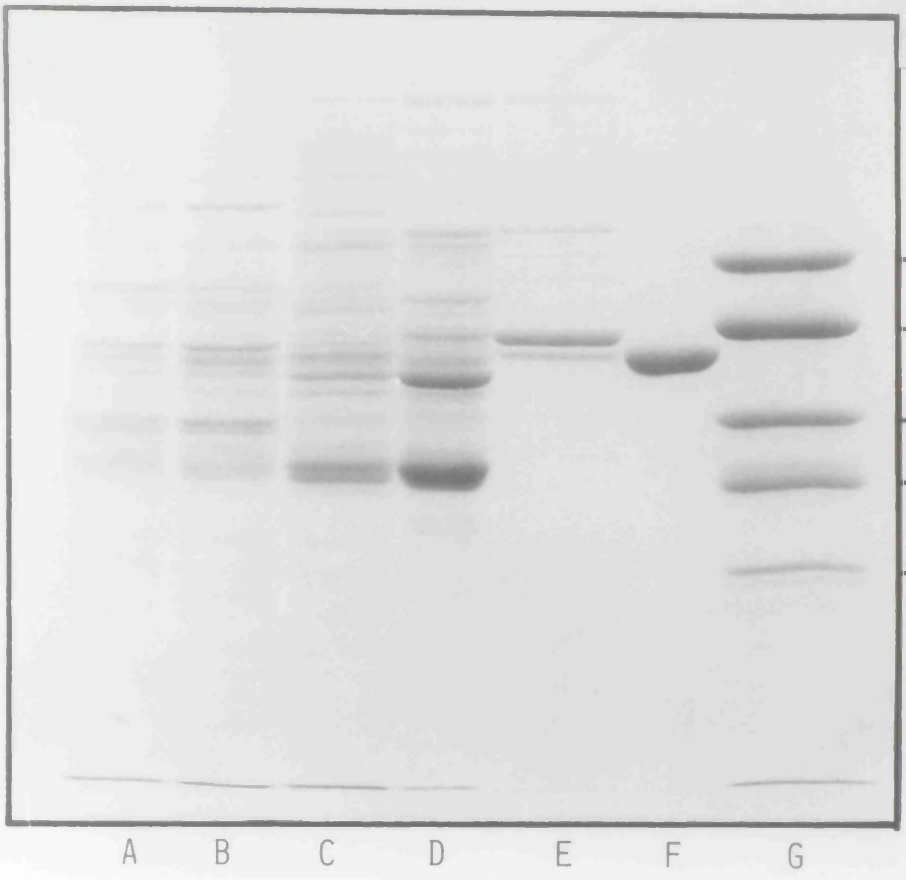


Figure 3.3 Purification of chorismate synthase from N. crassa

SDS PAGE (10%) monitoring the purification of chorismate synthase from N. crassa. Track A, crude extract; track B, negative DE52 DEAE-cellulose chromatography; track C, 40-50% $(NH_4)_2SO_4$ fraction; track D, DEAE-Sephacel pool; track E, phosphocellulose pool; track F, enzyme eluted from Mono Q column (8ug); track G, M_r markers.

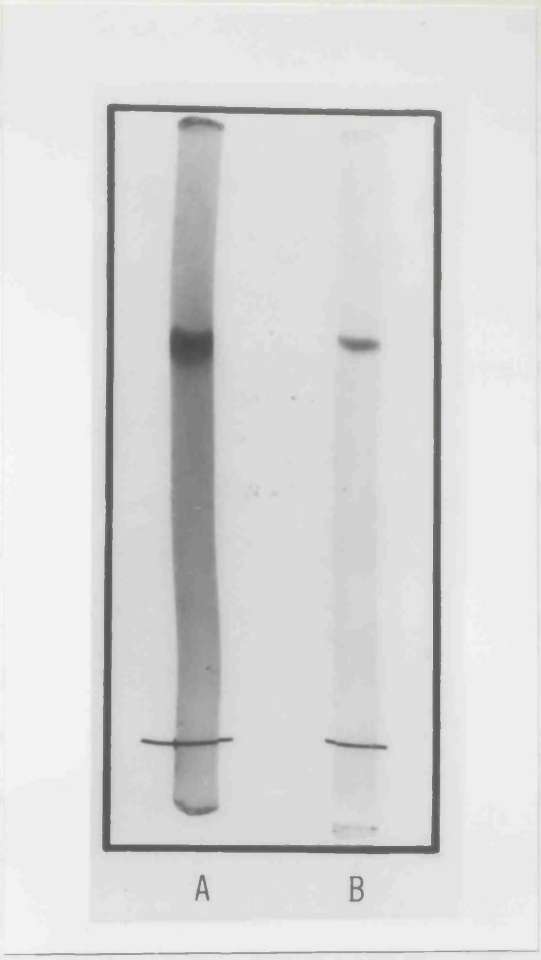


Figure 3.4 Diaphorase activity staining

These non-denaturing 5% tube gels of the purified N. crassa chorismate synthase were stained (A) for diaphorase activity and (B) for protein as described in the Materials and Methods section. (A) and (B) were both loaded with 8ug of highly purified N. crassa chorismate synthase.

performance chromatography in conjunction with chemical crosslinking techniques were used to determine the native M_r. The subunit M_r of N. crassa chorismate synthase was determined by SDS PAGE.

3.5.1 Subunit M_r of the N. crassa chorismate synthase

The subunit M_r of the purified enzyme was determined under denaturing conditions by comparing electrophoretic mobility with the mobility of standard proteins of known M_r (Figure 3.5). Comparison of the mobility of chorismate synthase with these standards gave a subunit M_r of 50 000.

3.5.2 Native M_r of the N. crassa chorismate synthase by gel permeation chromatography

Standard proteins of known M_r were used to calibrate both a conventional sephacryl S300 gel filtration column and a Superose 6 gel filtration column which was run on a Pharmacia f.p.l.c. apparatus. Standard curves of peak V_e against molecular weight were constructed (Figure 3.6 and Figure 3.7). The measured peak V_e for chorismate synthase corresponded to a M_r of 195 000 on the S300 column and 198 000 on the Superose 6 column. Gel filtration using f.p.l.c produced identical peak elution volumes for purified enzyme and for chorismate synthase activity in 100 000g supernatants of N. crassa cell extracts.

3.5.3 Native M_r of the N. crassa chorismate synthase by chemical crosslinking

Confirmation of the oligomeric nature of the N. crassa chorismate synthase was made by crosslinking with dimethyl suberimidate (Davies & Stark, 1970). Crosslinked forms of the enzyme were separated by SDS PAGE (Figure 3.8) and a densitometer scan of the Coomassie Blue-stained gel (Figure 3.9) used to

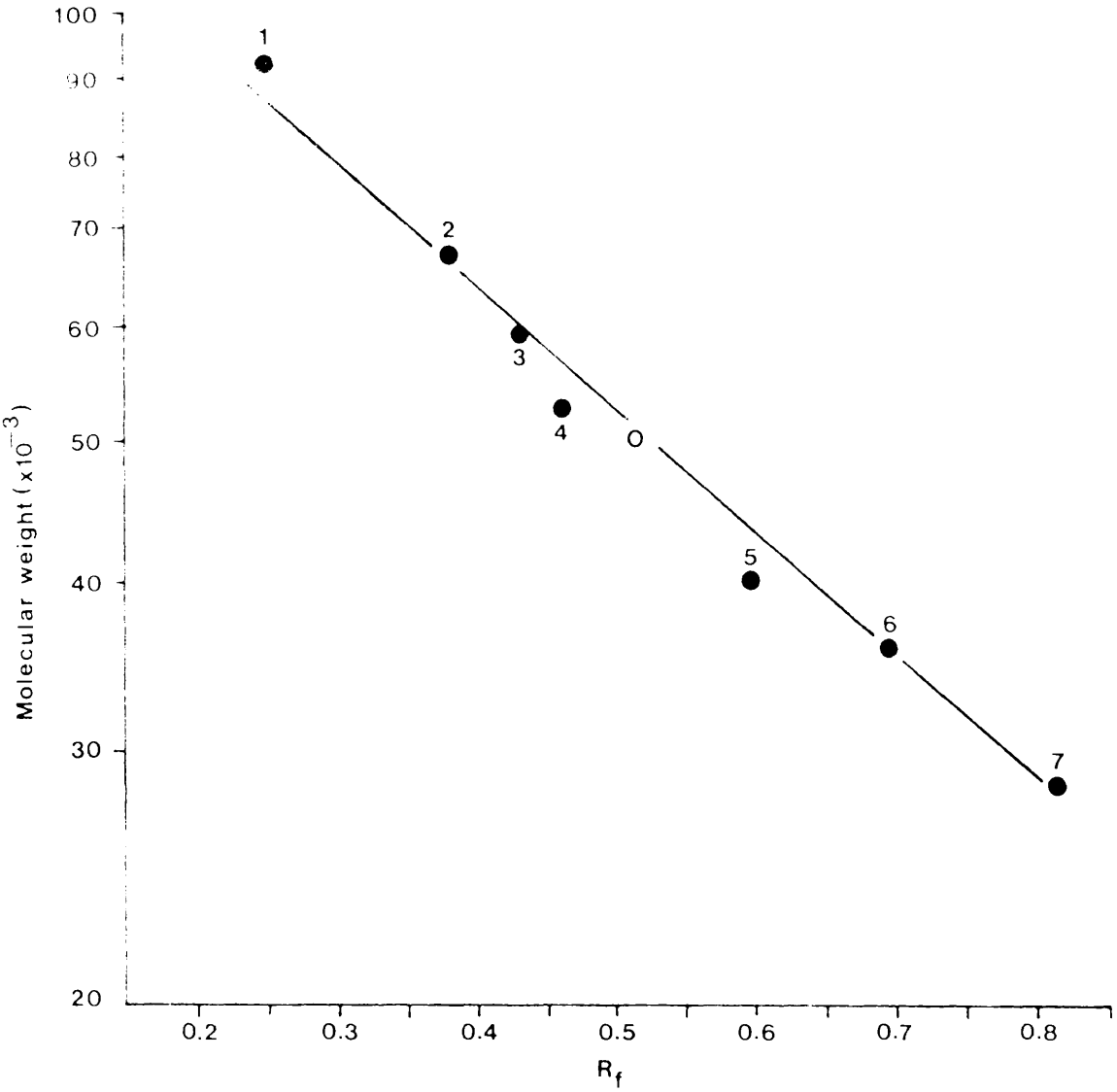


Figure 3.5 SDS PAGE standard curve

SDS PAGE (10%) was performed as described in Section 2.3. The R_f values of standard proteins (Section 2.8.1) were calculated and plotted against their molecular weight. The R_f of the purified N. crassa chorismate synthase is shown (o). The standard curve was fitted by eye.

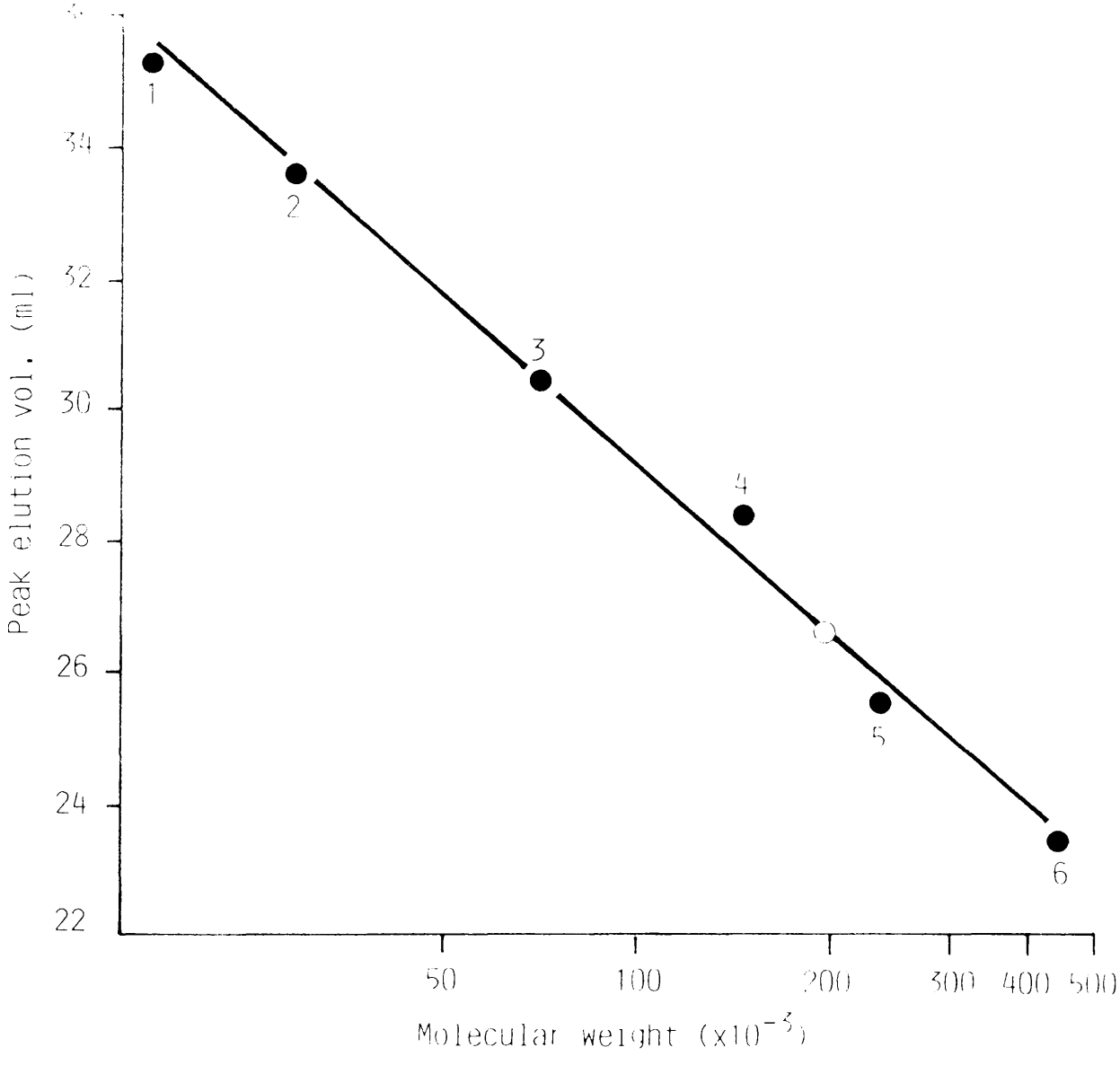


Figure 3.6 S300 gel filtration standard curve

A S300 gel filtration column was calibrated and run as described in Section 2.8.2(a). Peak elution volume (ml) was plotted against molecular weight. (●) standard proteins; (○) purified N. crassa chorismate synthase. The standard curve was fitted by eye.

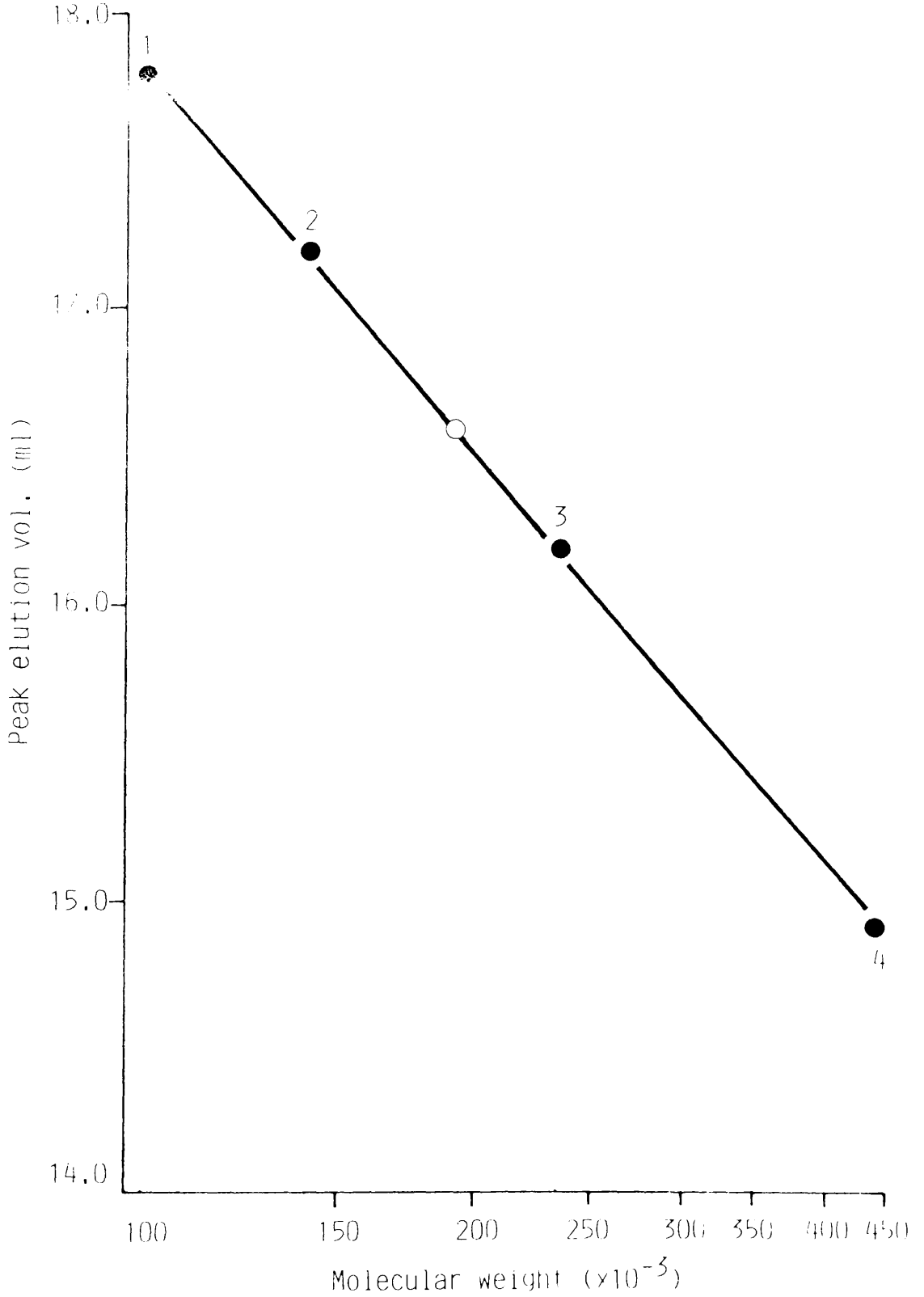


Figure 3.7 Superose 6 gel filtration standard curve

A Superose 6 gel filtration column was calibrated and and run as described in Section 2.8.2(b). Peak elution volume (ml) was plotted against molecular weight. (●) standard proteins; (○) purified *N. crassa* chorismate synthase. The standard curve was fitted by eye.

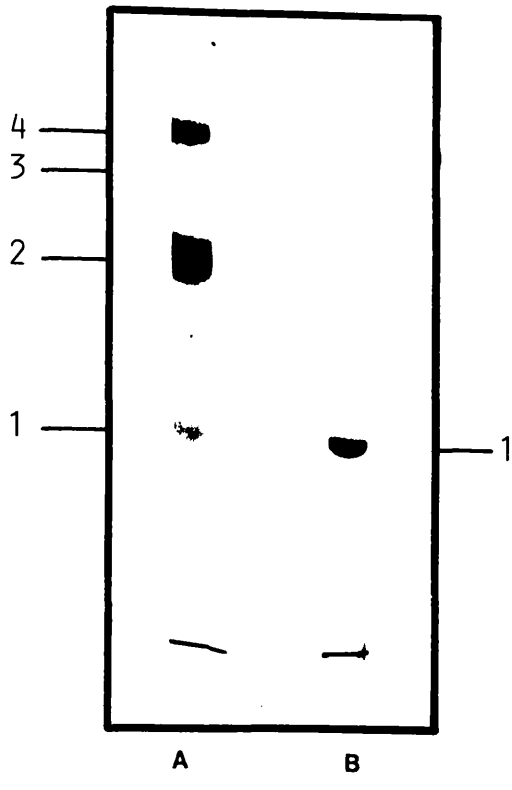


Figure 3.8 N. crassa chorismate synthase cross-linked with dimethylsuberimidate

These 5% gels were run as described in Section 2.9 and stained for protein. Track A, 36ug of crosslinked N. crassa chorismate synthase; track B, 10ug of N. crassa chorismate synthase.

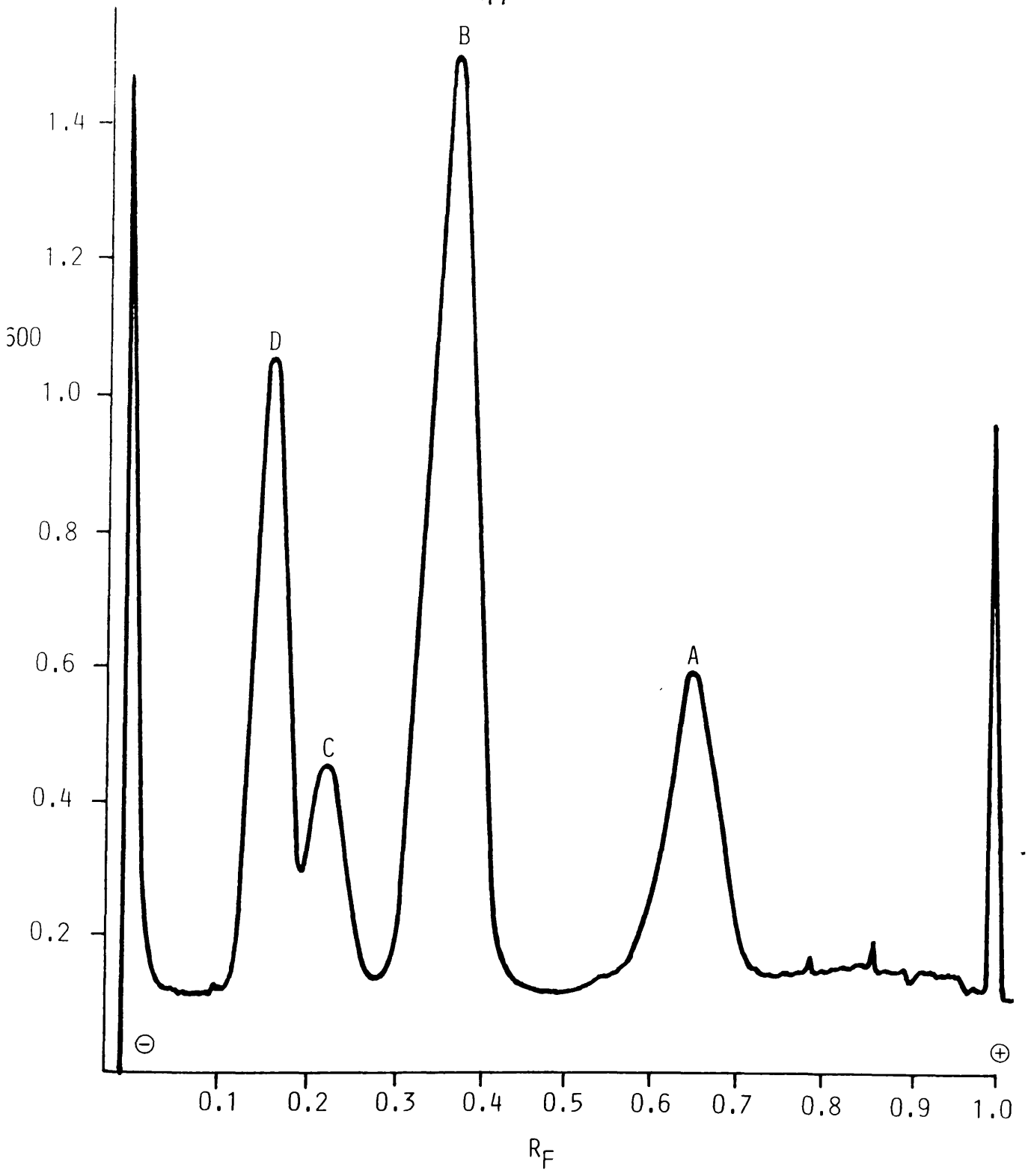


Figure 3.9 Densitometer scan of the N. crassa chorismate synthase crosslinked with dimethylsuberimidate. (Tube gel A in Figure 3.8)

The four peaks labelled A, B, C and D correspond to monomeric, dimeric, trimeric and tetrameric species of the enzyme.

determine their electrophoretic mobilities. A standard curve of subunit M_r against electrophoretic mobility was constructed using uncrosslinked marker proteins and the M_r of the crosslinked subunit species estimated (Figure 3.10, Table 3.2).

3.6 Activity staining of the N. crassa chorismate synthase

The purified N. crassa chorismate synthase has an intrinsic flavin reductase (diaphorase) activity. Native PAGE of the enzyme shows a single protein band which co-migrates with a single diaphorase activity band (Figure 3.4).

3.7 Discussion

An improved purification procedure has been developed that reproducibly gives homogeneous N. crassa chorismate synthase of high specific activity. The enzyme required to be purified over 3000 fold from crude extract. The purification procedure is outlined in Table 3.1 and a polyacrylamide gel showing the protein components at each stage is shown in Figure 3.3. The purified enzyme had a specific activity of 32.1 units/mg protein. To ensure the stability of the enzyme throughout the purification DTT was present in all buffers and a full antiprotease strategy was employed. This strategy comprised of three main elements; (1) The inclusion of 1.2mM-PMSF in all buffers at all stages throughout the purification. PMSF is a powerful serine endopeptidase inhibitor (Yu et al., 1973). (2) A 'negative' chromatographic step on DE52 to bind four of the major intracellular proteases found in N. crassa (Siepen et al., 1975). (3) The addition of benzamidine during $(NH_4)_2SO_4$ fractionation. Benzamidine is an effective

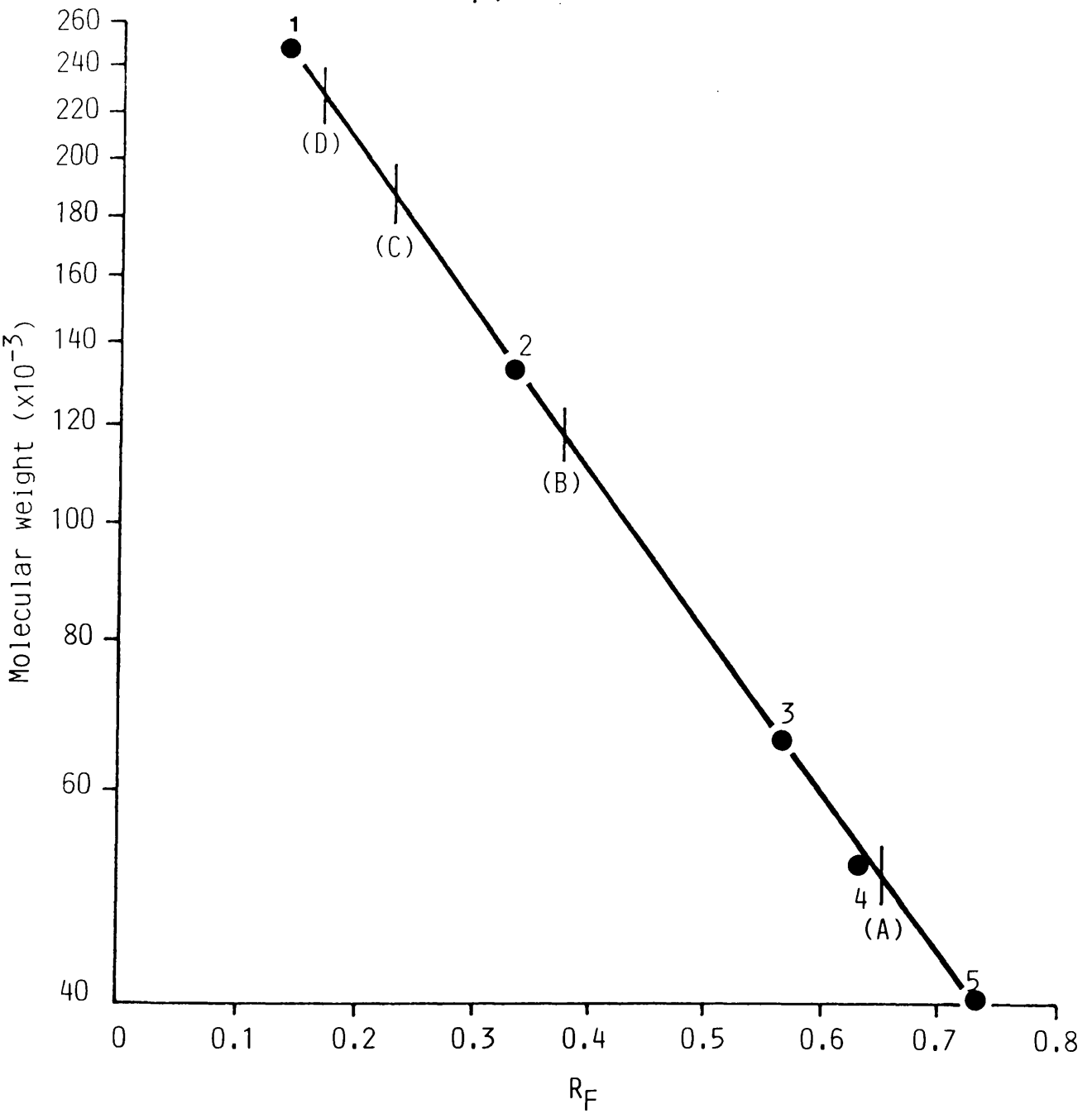


Figure 3.10 SDS PAGE standard curve

SDS PAGE (5%) was performed as described in Section 2.3. The R_f values of standard proteins were calculated and plotted against their molecular weight. The R_f values of the crosslinked species of N. crassa chorismate synthase are shown by the four vertical lines labelled A, B, C and D. Standard proteins were: 1, fatty-acid synthase (M_r 250 000); 2, β-galactosidase (M_r 116 000); 3, bovine serum albumin (M_r 68 000); 4, glutamate dehydrogenase (M_r 53 000); 5, aldolase (M_r 40 000). The standard curve was fitted by eye.

TABLE 3.2

Oligomeric forms of crosslinked N.crassa chorismate synthase

Crosslinked species	oligomeric form	predicted M_r n x 50 (Where n = no. of subunits)	M_r determined from calibration curve (Figure 3.8)
A (1)	monomer	50	50
B (2)	dimer	100	118
C (3)	trimer	150	185
D (4)	tetramer	200	230

reversible inhibitor of trypsin like proteases (Mares-Guira & Shaw, 1965) and unlike PMSF is not salted out at high concentrations of $(NH_4)_2SO_4$. Because of the severity of the protease problem in N. crassa it was essential this strategy was adhered to strictly. The enzyme could not be stored safely at any stage and purifications of minimum duration gave the highest yields.

Three chromotographic steps were required to produce homogeneous enzyme. The first of these was anion-exchange chromatography. Chorismate synthase activity was bound to a column of DEAE-Sephacel and eluted with a linear gradient of salt. Chorismate synthase activity eluted at a salt concentration of 90mM. At this stage in the purification chorismate synthase was only a minor component of a complex mixture of polypeptides (Figure 3.3, track D). The pooled active fractions from this step were then dialysed into a low phosphate buffer, pH6.5 and subject to cation-exchange chromatography on cellulose phosphate. Phosphocellulose chromatography is a major purification step in previous work on this enzyme (Cole & Gaertner, 1975; Boocock, 1983) and on chorismate synthase from B. subtilis (Hasan & Nester, 1978). The enzyme eluted from cellulose phosphate with the second of two linear gradients of potassium phosphate (100-400mM). Phosphocellulose chromatography is one of two major purification steps in this improved procedure. There is a ten fold increase in chorismate synthase specific activity after phosphocellulose chromatography (Table 3.1) and SDS PAGE of the pooled fractions shows the enzyme to be one of only four major and several minor components (Figure 3.3, track E). The final purification step was chromatography on Mono Q using a Pharmacia f.p.l.c apparatus. The

combination of a low pH Bis-Tris/HCl buffer and the high resolving power of the anion-exchange column resulted in a further ten-fold increase in chorismate synthase specific activity and allowed the enzyme to be separated from other contaminating polypeptides. Purified chorismate synthase was stable for at least two months when stored in 50% glycerol at -20^{o}C.

The specific activities of other chorismate synthases in the purified form and in crude cell free extracts are compared with the enzyme in this study (Table 3.3 and Table 3.4). The specific activity of the N. crassa chorismate synthase in crude extracts compares favourably with the specific activities of the other characterised enzymes. The specific activities reported reflect the low levels of abundance of chorismate synthase found in these organisms. The specific activity of the purified N. crassa chorismate synthase from this study is twice that of the estimated specific activity from the best previous purification (Boocock, 1983) and fifteen times that of the " > 90% homogeneous" chorismate synthase reported by Welch et al. (1974), although differences in assay conditions must be taken into account.

The detailed structural analysis of the purified enzyme from this study indicates that the N. crassa chorismate synthase is a tetrameric protein of subunit M_r 50 000. Each polypeptide possesses both chorismate synthase and diaphorase activity and the enzyme can be considered bifunctional. Native M_r values obtained from conventional and f.p.l.c gel filtration studies agree with chemical crosslinking experiments and SDS PAGE.

Chorismate synthases from other sources show a diversity of quaternary and subunit M_r values. Table 3.5 shows a comparison of subunit and native M_r values determined for these enzymes.

TABLE 3.3

Specific activities of purified chorismate synthases

Source	Reference	Assay conditions	Specific activity (units/mg)
N. crassa	This study	25°C, 50mM-TEA•HCl-KOH pH7.0, 50mM KCl, 50uM EPSP, 20 uM NADPH, 10uM FMN	32.1
N. crassa	Boocock (1983)	25°C, 50mM-TEA•HCl-KOH pH7.0, 50mM KCl, 2.5mM $MgCl_2$, 50uM EPSP, 20uM NADPH, 10uM FMN	15.0 (est)
N. crassa	Welch *et al.*(1974)	25°C, 100mM-potassium phosphate pH7.0, 1mg/ml BSA, 50uM EPSP 50uM NADPH, 10uM FMN	0.7
B. subtilis	Hasan & Nester (1978)	37°C, 2mM Tris-HCl, pH7.2, 50uM EPSP, 50uM NADPH, 10uM FMN, 0.33mM EDTA, 2mM $MgCl_2$, 10mM glutamine, 3mM B-Me, 2mg/ml BSA, 0.02U Anthranilate synthase, 0.075U Flavin reductase.	0.92

TABLE 3.4

Specific activities of chorismate synthases in cell-free extracts

Source	Reference	Assay conditions	Specific activity (units/mg)
N. crassa	This study	see Table 3.3	0.01
N. crassa	Lewendon (1984)	as Boocock (1983) in Table 3.3	0.005
B. subtilis	Hasan & Nester (1978)	see Table 3.3	0.0066
E. coli B-37	Morell *et al.* (1967)	37°C, 100mM-Tris-HCl pH8.0, 1mM EPSP, 1mM NADH, 0.2mM FAD, 5mM $MgCl_2$, 5mM DTT, *E. coli* NADH dehydrogenase 0.1-0.2mg, *E. coli* chorismate mutase/prephenate dehydratase 0.1-0.2mg. Anaerobic assay.	0.022
E. coli K12	This study	25°C, 50mM-potassium phosphate pH7.0, 50uM EPSP, 10uM FAD, 2mM sodium dithionite.	0.013 (0.025)[1]
P. sativum	Mousdale & Coggins (1987)	25°C, 50mM-Bis Tris-HCl pH7.0, 50mM KCl, 200uM EPSP, 10uM FMN, 10mM L-glutamine, 5mM $MgCl_2$, 60 p kat/ml *N. crassa* Anthranilate synthase.	0.0036

1. Value in presence of 10uM FMN

TABLE 3.5

Subunit sizes and quaternary structures of chorismate synthases

Source	Reference	Subunit size M_r (KDa)	Quaternary Structure
N. crassa	This study	50	Tetramer
N. crassa	Welch *et al* (1974)	55	Dimer
E. coli	This study	38	Tetramer
B. subtilis	Hasan & Nester (1978)	24 (13)[1] (17)[2]	

1. Diaphorase subunit
2. Dehydroquinate synthase

Welch et al. (1974) claimed the N. crassa chorismate synthase existed in at least two multimeric states, the most stable being a dimeric form with subunit M_r 55 000. The material purified in this study showed no such structural heterogeneity, and in view of the unrecognised protease problem at that time the existence of multimeric states of the enzyme may well reflect proteolytic damage. The B. subtilis enzyme is found to exist in a trifunctional enzyme complex along with a flavin reductase (diaphorase) activity and another of the shikimate pathway enzymes, dehydroquinate synthase (Hasan & Nester, 1978a,b,c). Protein-protein interactions between the subunits appeared to be important in maintaining catalytic activity. The E. coli chorismate synthase has been purified from an overproducing strain (see Chapter 4) and the amino acid sequence determined (White et al., 1987). The enzyme appears to exist as a monofunctional chorismate synthase subunit with a tetrameric quaternary structure (see Chapter 4). Possible reasons for the differences in the subunit M_r and structural organisation of these enzymes will be discussed later in this study.

3.8 Summary

These results indicate that the N. crassa chorismate synthase purified by this procedure is a tetrameric protein with a subunit M_r of 50 000. Each polypeptide chain appears to be bifunctional, carrying both chorismate synthase and flavin reductase (diaphorase) activity. The specific activity of the homogeneous enzyme is significantly greater than that of previously purified N. crassa chorismate synthases.

CHAPTER 4 THE PURIFICATION AND PRELIMINARY CHARACTERISATION OF THE E. COLI CHORISMATE SYNTHASE FROM THE OVERPRODUCING STRAIN AB2849/pGM602

4.1 Introduction

The purification and characterisation of the E. coli chorismate synthase has been hindered by a combination of the low levels at which it occurs and its requirement for strict anaerobic assay conditions. Previous purification attempts relied on rigorous degassing procedures to assay the enzyme and resulted in only partially purified material (Morell et al, 1967). These problems were solved by the cloning and overexpression of the E. coli aroC gene which encodes chorismate synthase (Millar et al, 1986) and the development of a simple anaerobic assay for the enzyme (White et al, 1987).

Hagervall & Björk (1984) located the aroC gene near the trmC gene on the E. coli chromosome. The Clarke and Carbon plasmid pLC33-1 (Clarke & Carbon, 1979) was identified as carrying the region of the E. coli genome near the aroC gene. The presence of the aroC gene within this plasmid was confirmed by its ability to relieve the auxotrophic requirements of the E. coli K12 aroC$^-$ mutant AB2849 (Pittard & Wallace, 1966). The aroC gene was subcloned from pLC33-1 as a 5.3 kbp ClaI fragment in the multicopy plasmid pAT153 (Twigg & Sherratt, 1980). The DNA insert in this plasmid, was then subcloned and a smaller plasmid pGM602 containing the aroC gene on a 1.65kb insert was obtained (Millar et al, 1986). This plasmid was used to transform the aroC$^-$ strain

of E. coli. The transformed strain designated E. coli AB2849/pGM602 synthesised elevated levels of chorismate synthase (Millar et al, 1986). The complete nucleotide sequence of the gene has been determined and the N-terminal portion of the aroC gene product confirmed (see Chapter 5).

This chapter describes the partial purification of chorismate synthase from E. coli K12 'wild-type' and E. coli AB2849/pGM602, the development of a simple anaerobic assay for chorismate synthase and the subsequent purification of the overproduced enzyme. Some preliminary structural analysis is also presented.

4.2 A partial purification of chorismate synthase from E. coli K12 and E. coli AB2849/pGM602

4.2.1 Purification procedure

A 20g batch of E. coli AB2849/pGM602 cells was suspended in 12ml of 100mM-potassium phosphate buffer pH7.0, containing 1.2mM-PMSF, 5mM-EDTA, 1mM-benzamidine and 0.4mM-DTT and broken by two passages through a French pressure cell. The extract was diluted to 80ml, treated with DNAse(I) for 1h at $4^{o}C$ and then centrifuged at 28000g for 30 minutes to give the crude extract. This crude extract was then subjected to ammonium sulphate fractionation. The material precipitating between 28 and 45% saturation was resuspended in 8ml of extraction buffer and dialysed overnight against 1 litre of 20mM-Tris/HCl buffer pH7.6, containing 1.2mM-PMSF, 1mM-benzamidine and 0.4mM-DTT. 0.5ml of this dialysed protein was subjected to anion-exchange chromatography on a Mono Q column in a Pharmacia f.p.l.c apparatus. The enzyme was eluted with a linear gradient of salt (0 to 1M-NaCl, 0.5ml

fractions),(Figure 4.1). Chorismate synthase activity was found to elute at a salt concentration of 330mM.

A similar scheme was used for the purification of chorismate synthase from a 20g batch of 'wild-type' E. coli K12 cells. The 'wild-type' chorismate synthase activity eluted from the Mono Q column at the same salt concentration as the overproduced enzyme.

4.2.2 Detection of chorismate synthase activity

Chorismate synthase was detected at 25°C by monitoring the appearance of chorismate continuously in a spectrophotometric assay at 275nm. The assay mixture (total volume 1ml) contained (final concetrations) 50mM-TEA HCl/KOH buffer pH7.0, 50mM-KCL, 2.5mM-$MgCl_2$, 10uM-FAD and 50uM-EPSP. To this cocktail was added sufficient dithionite to reduce all flavin and to remove all the dissolved oxygen. The difficulty in monitoring the appearance of chorismate in the presence of sodium dithionite which absorbs strongly in the U.V. did not allow accurate and reproducible measurements of chorismate synthase activity. Enzyme activity could be detected in crude extracts of the overproducing strain of E. coli but the spectrophotometric assay was not of sufficient sensitivity to detect 'wild-type' K12 levels in similar crude extracts.

4.2.3 SDS PAGE of partially purified chorismate synthase from E. coli AB2849/pGM602 and E. coli K12

A prominent feature in SDS polyacrylamide gels of the partially purified enzyme obtained from the overproducing strain was a band of subunit M_r 40 000. This band, which was present only in trace amounts in the preparations obtained by a similar procedure

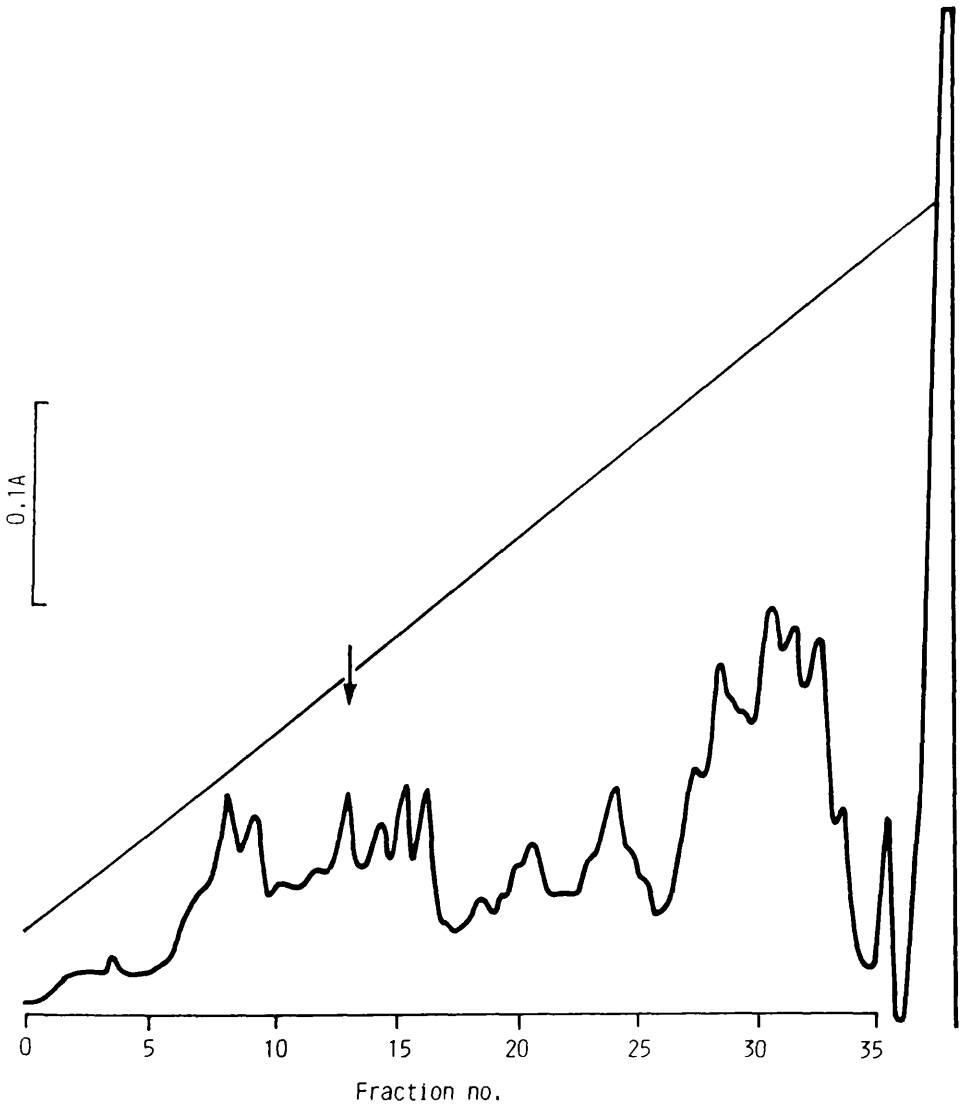


Figure 4.1 Chromatography of proteins from E. coli AB2849/pGM602 on Mono Q

12 mg of protein in 0.5ml of 20mM-Tris/HCl, pH7.5 was applied to a Pharmacia Mono Q column and eluted with a zero to 1M gradient of NaCl. The trace shows the absorbance of 280nm. Flow rate 1ml/min, 0.5ml fractions. The arrow indicates the fraction containing peak chorismate synthase activity.

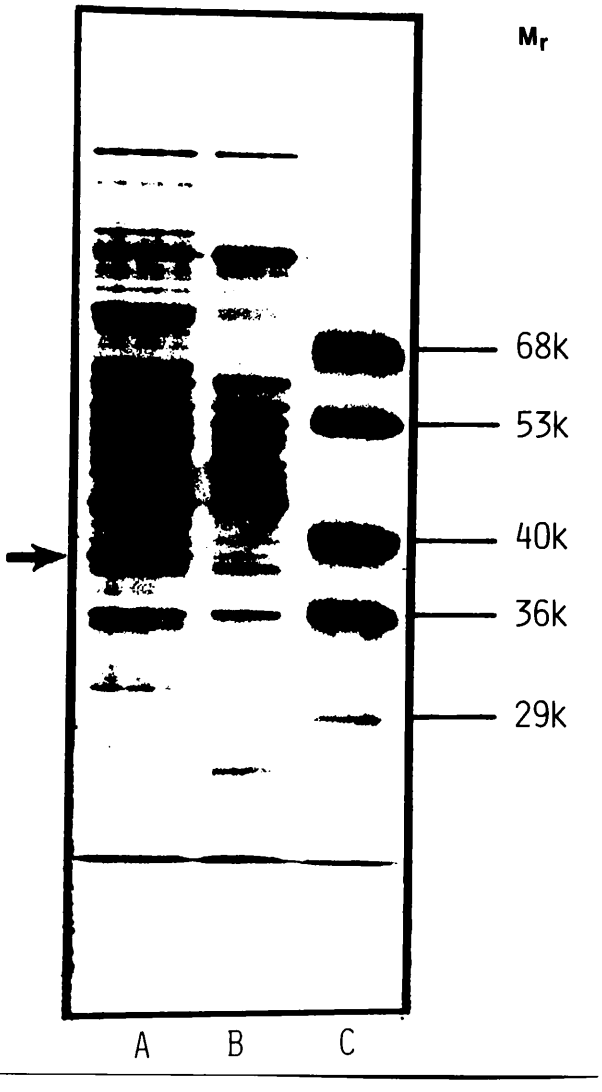


Figure 4.2 SDS PAGE of partially purified chorismate synthase

This 10% Laemlli gel was stained with silver nitrate.
Track A: partially purified enzymes from AB2849/pGM602 (10ug)
Track B: partially purified enzyme from wild type K12 E. coli (10ug)
Track C: molecular weight markers
The band tentatively identified as chorismate synthase is indicated with an arrow.

from 'wild-type' cells, was tentatively identified as chorismate synthase (Figure 4.2, track A). The subunit M_r of 40 000 is consistent with the size of the genomic insert in pGM602 (1.65kbp).

4.3 Development of a simple anaerobic assay for chorismate synthase

4.3.1 Detection of chorismate using h.p.l.c.

The continuous spectrophotometric assay used in the partial purification of E. coli chorismate synthase lacked sensitivity and reproducibility. The elevated levels of enzyme in E. coli AB2849/pGM602 were used to develop an assay procedure that was (1) simple, (2) sensitive and (3) reproducible. In the new assay procedure the formation of chorismate is measured directly following separation of the reaction products on a 'fast organic acids' cation h.p.l.c column. Anaerobic conditions are maintained via reduced flavin generated by the action of sodium dithionite on either FAD or FMN. Chorismate is retarded on Aminex HPX-87H Organic Acids Analysis columns (Mousdale & Coggins, 1985) and can therefore be readily separated from the substrate EPSP and other components required in the assay. As little as 0.2nmoles of chorismate may be accurately determined at 215nm using this method. Separation and detection of product can be achieved in less than 15 min. The amount of product formed is measured by comparing the peak area with that of a standard sample of chorismate.

4.3.2 Precise assay conditions

The standard 1ml assay contains 50mM-potassium phosphate buffer pH7.0, 50uM-EPSP, 10uM-FAD or FMN and 2mM-sodium dithionite. To initiate an assay an aliquot of enzyme is added to

a cocktail containing all of the above components in a 1ml assay cuvette. The reaction vessel is stoppered and incubated in a water bath at 25°C. 20ul aliquots of the reaction mixture are removed at various timepoints and immediately chromatographed on a Biorad Fast Organic Acids Analysis column (100mm x 7.8mm; flow rate 1.0ml/min; solvent 40mM-H_2SO_4). A typical chromatographic trace is shown in Figure 4.3 and the linearity of the assay with respect to time is demonstrated in Figure 4.4.

This new assay procedure was used to purify the E. coli chorismate synthase from the overproducing strain AB2849/pGM602 This procedure is described in the next section.

4.4 Purification procedure

All steps after the breaking of the cells were performed at 4°C.

Step 1: Extraction and centrifugation. A 20g batch of E.coli (strain AB2849/pGM602) was suspended in 10ml of 50mM-Tris/HCl buffer, pH7.5, containing 0.4mM-DTT and 1.2mM-PMSF (Buffer A) and broken by two passages through a French pressure cell. The extract was diluted to 80ml with the above buffer and 0.5mg DNAase(I) was added. After 1h stirring the resulting suspension was centrifuged at 28 000g for 30min. Chorismate synthase was purified from the resulting supernatant.

Step 2: Fractionation with $(NH_4)_2SO_4$. The crude extract was made 1mM in benzamidine and adjusted carefully to 35% saturation with solid $(NH_4)_2SO_4$ (208g/l). After stirring for 20min the precipitate was removed by centrifugation at 28 000g for 30min. The supernatant was adjusted to 60% saturation (390g/l) and stirred

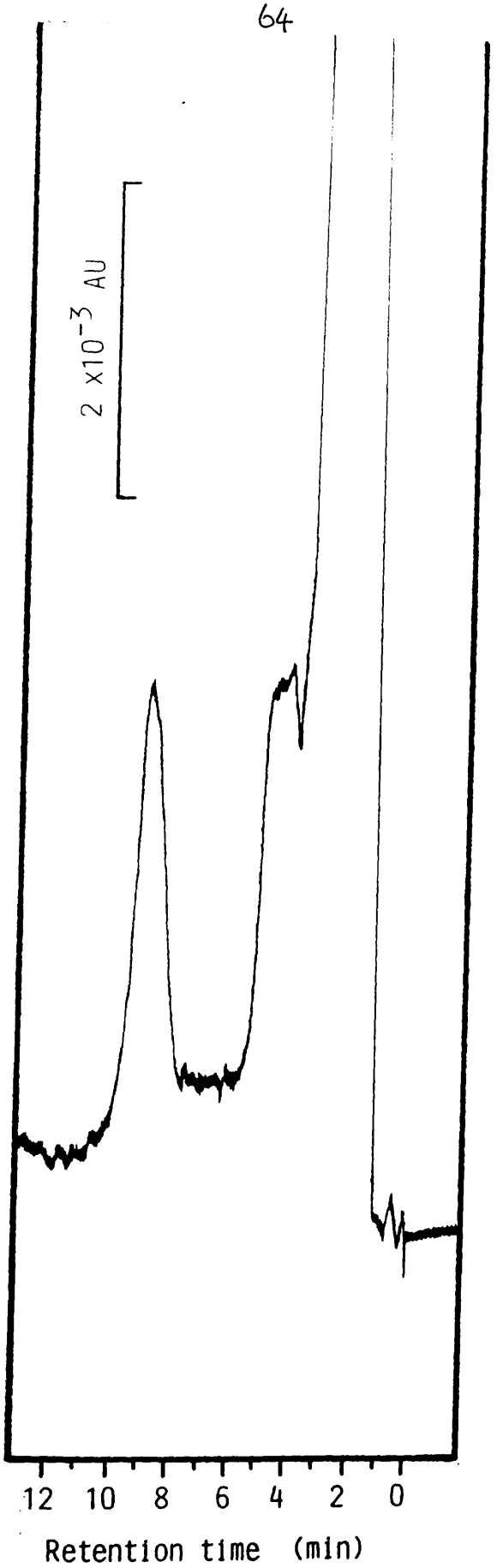


Figure 4.3 Separation of chorismate using h.p.l.c.

Column: Biorad Aminex HPX-87H
Chorismic acid elutes from the column at 9 minutes

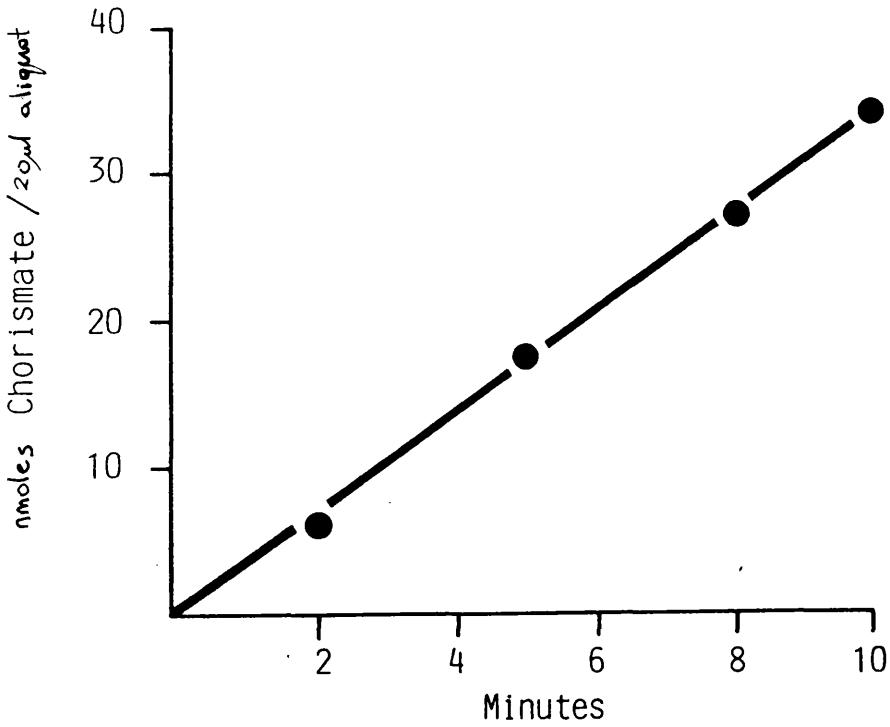


Figure 4.4 Linearity of the anaerobic assay procedure

Aliquots (20µl) were removed from the assay of various times and the amount of chorismate determined by h.p.l.c. analysis. In this experiment each assay contained 1.5 mg of protein from a crude extract of E. coli strain AB2849/pGM602, 10uM FAD, 50uM EPSP and 2mM dithionite.

for 20min. The precipitated protein was collected by centrifugation at 28 000g resuspended in buffer A and dialysed overnight against 2 litres of 50mM-Tris/HCl buffer, pH7.5, containing 0.4mM-DTT, 1.2mM-PMSF and 50mM-KCl (buffer B).

Step 3: DEAE-Sephacel chromatography. The dialysed material was loaded onto a column of DEAE-sephacel (bed volume 60ml) equilibrated in buffer B. The column was washed with buffer until the A_{280} of the eluate had fallen to a value below 0.3. The column was then eluted with a 400ml linear gradient of 50-250mM-KCl in 50mM-Tris/HCl buffer, pH7.5 containing 0.4mM-DTT (flow rate 70ml/h). Fractions (5ml) containing chorismate synthase activity were pooled and dialysed overnight against 2x2 litres of 10mM-potassium phosphate buffer, pH6.6, containing 0.4mM-DTT (buffer C).

Step 4: Cellulose phosphate (P11) chromatography. The dialysed extract was loaded onto a column (bed volume 25ml) of cellulose phosphate equilibrated in buffer C and washed with the same buffer until the A_{280} of the eluate had fallen to a value below 0.05. The column was then eluted with a 400ml linear gradient of 10-400mM-potassium phosphate buffer, pH6.6 containing 0.4mM dithiothreitol (flow rate 20ml/h). Fractions (5ml) containing chorismate synthase activity were pooled and dialysed overnight against 2x1 litre of 50mM-Tris/HCl buffer containing 0.4mM-DTT. The protein was concentrated by adsorption on a 1ml (bed volume) DEAE-Sephacel column and eluted with the above buffer containing 1.0-M KCl. The concentrated enzyme was then dialysed overnight against 500ml 50mM-Tris/HCl buffer pH7.5, containing 0.4mM-DTT and 0.8M-$(NH_4)_2SO_4$.

Step 5: Phenyl-sepharose chromatography. The dialysed material was loaded onto a column of phenyl-sepharose (bed volume 1ml) equilibrated in 50mM-Tris/HCl buffer pH7.5, containing 0.4mM-DTT and 0.8M-$(NH_4)_2SO_4$. Chorismate synthase activity was eluted with a decreasing linear gradient of $(NH_4)_2SO_4$ (0.8-0.0M) in the above buffer (flow rate 10ml/h). Fractions (1.5ml) containing chorismate synthase activity were pooled and dialysed against 1 litre 50mM-Tris/HCl buffer, pH7.5 containing 0.4mM-DTT. The enzyme was concentrated by adsorption on a 1ml (bed volume) DEAE-sephacel column as before. The concentrated protein was dialysed against 50mM-Tris/HCl buffer, pH7.5 containing 0.4mM-DTT and 50% v/v glycerol and stored at -20°C.

4.5 Summary of the purification procedure

The results obtained from a typical purification procedure are shown in Table 4.1 and a SDS polyacrylamide gel showing the protein components at each stage is shown in Figure 4.5.

4.6 Purity of the E. coli chorismate synthase

SDS PAGE of the purified enzyme showed a single band when stained for protein (Figure 4.5, track E).

4.7 Molecular weight

The native M_r of E. coli chorismate synthase was determined by gel permeation chromatography and chemical crosslinking experiments. The subunit M_r was determined by SDS PAGE.

Table **4.1** Purification scheme for E. coli chorismate synthase

Tne results presented are for a typical purification starting from 20g of E.coli AB2849/pGM602 cells

	Step	Volume (ml)	Concn. of protein(mg/ml)	Total protein(mg)	Activity (units/ml)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
1:	Crude extract	60	27.5	1650	0.60	36	0.022	1	100
2:	35-60% $(NH_4)_2SO_4$	44	23.0	1012	0.69	30.5	0.030	1.4	85
3:	DEAE-Sephacel	44	4.0	176	0.50	22.0	0.125	5.7	61
4:	Cellulose phosphate	46	0.08	3.7	0.56	26.0	7.03	319	72
5:	Phenyl Sepharose	29	0.04	1.16	0.58	16.8	14.8	658	47

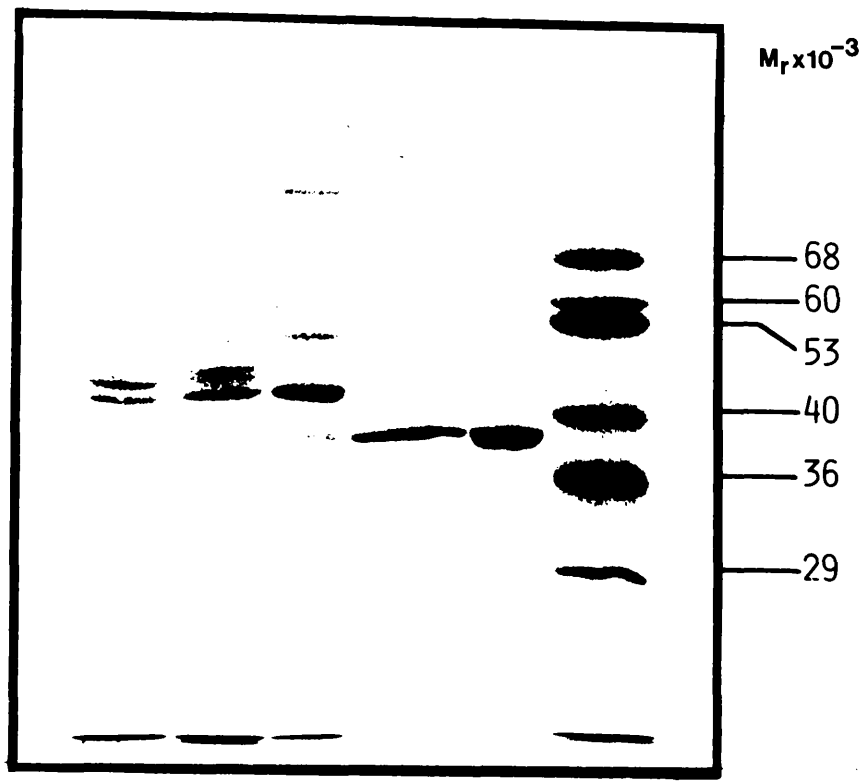


Figure 4.5 Purification of chorismate synthase from E. coli AB2849/pGM602

SDS PAGE (10%) monitoring the purification of chorismate synthase from E. coli. Track A, crude extract; track B, 35-60% $(NH_4)_2SO_4$ fraction; track C, enzyme eluted from DEAE-Sephacel, track D, enzyme eluted from phosphocellulose; track E, enzyme eluted from phenyl sepharose (10ug); track F, M_r markers.

4.7.1 Subunit M_r of the E. coli chorismate synthase

The mobility of the purified E. coli chorismate synthase was compared with mobilities of standard proteins of known M_r using SDS PAGE (Figure 4.6). Comparison with these markers gave a subunit M_r of 38 000.

4.7.2 Native M_r of the E. coli chorismate synthase by gel permeation chromatography

The native M_r of the E. coli chorismate synthase was determined by gel filtration on a Superose 6 column using a Pharmacia f.p.l.c apparatus. The column was calibtrated with proteins of known M_r and a standard curve of peak V_e against molecular weight was constructed (Figure 4.7). The measured peak V_e for E. coli chorismate synthase corresponded to an M_r of 144 000.

4.7.3 Native M_r of the E. coli chorismate synthase by chemical crosslinking

Confirmation of the quaternary structure of the E. coli chorismate synthase was made by crosslinking with dimethyl suberimidate (Davies & Stark, 1970). Crosslinked forms of the enzyme were separated by SDS PAGE revealing four distinct protein bands (Figure 4.8).

4.8 Discussion

A strain of E. coli which expresses elevated levels of chorismate synthase activity has been constructed (Millar, 1986). This strain (E. coli AB2849/pGM602) was used to develop a simple and reproducible anaerobic assay procedure for detecting chorismate

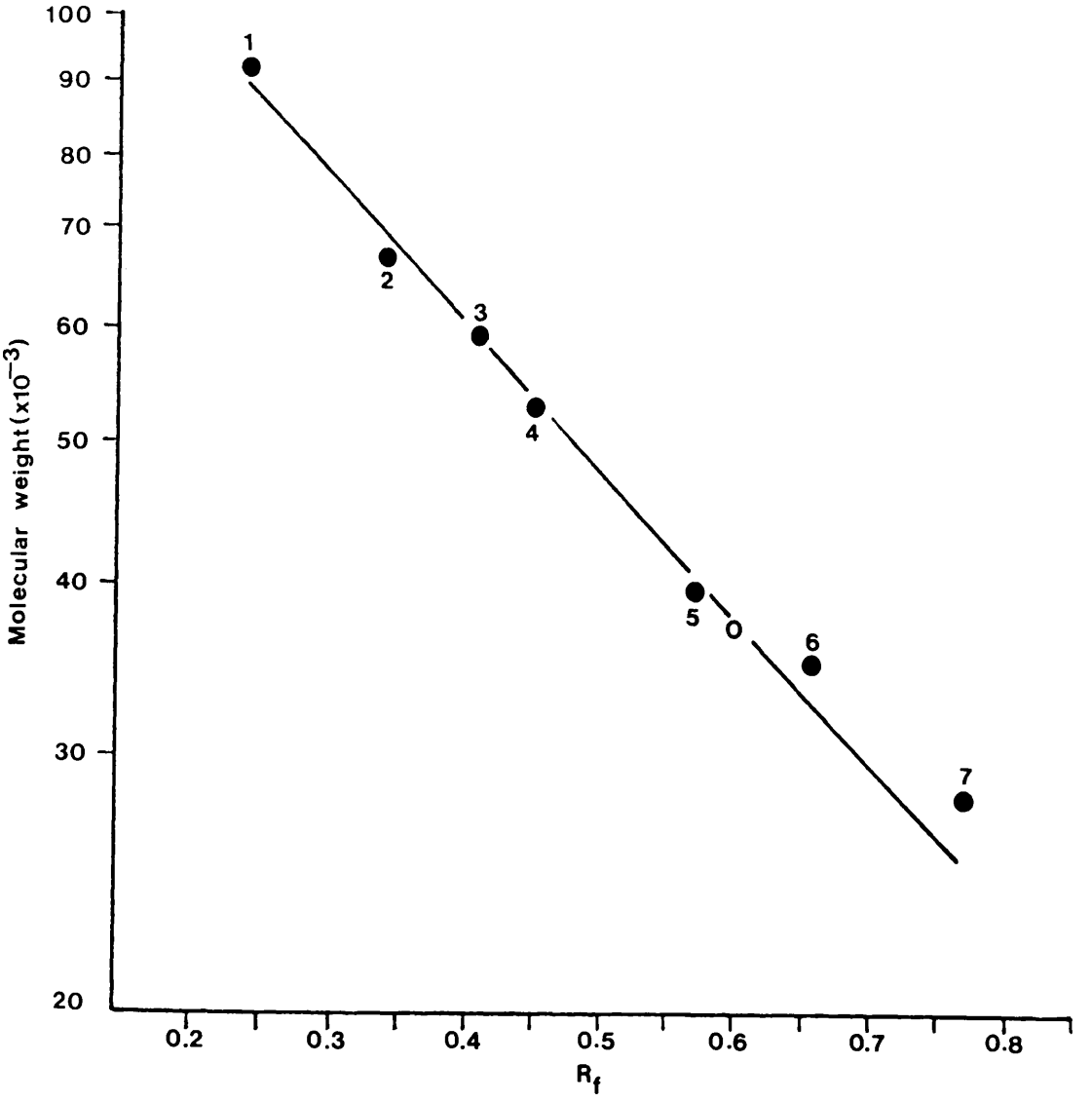


Figure 4.6 SDS PAGE standard curve

SDS PAGE (10%) was performed as described in Section 2.3. The R_f values of standard proteins (Section 2.8.1) were calculated and plotted against their molecular weight. The R_f of the purified E. coli chorismate synthase is shown (o). The standard curve was fitted by eye.

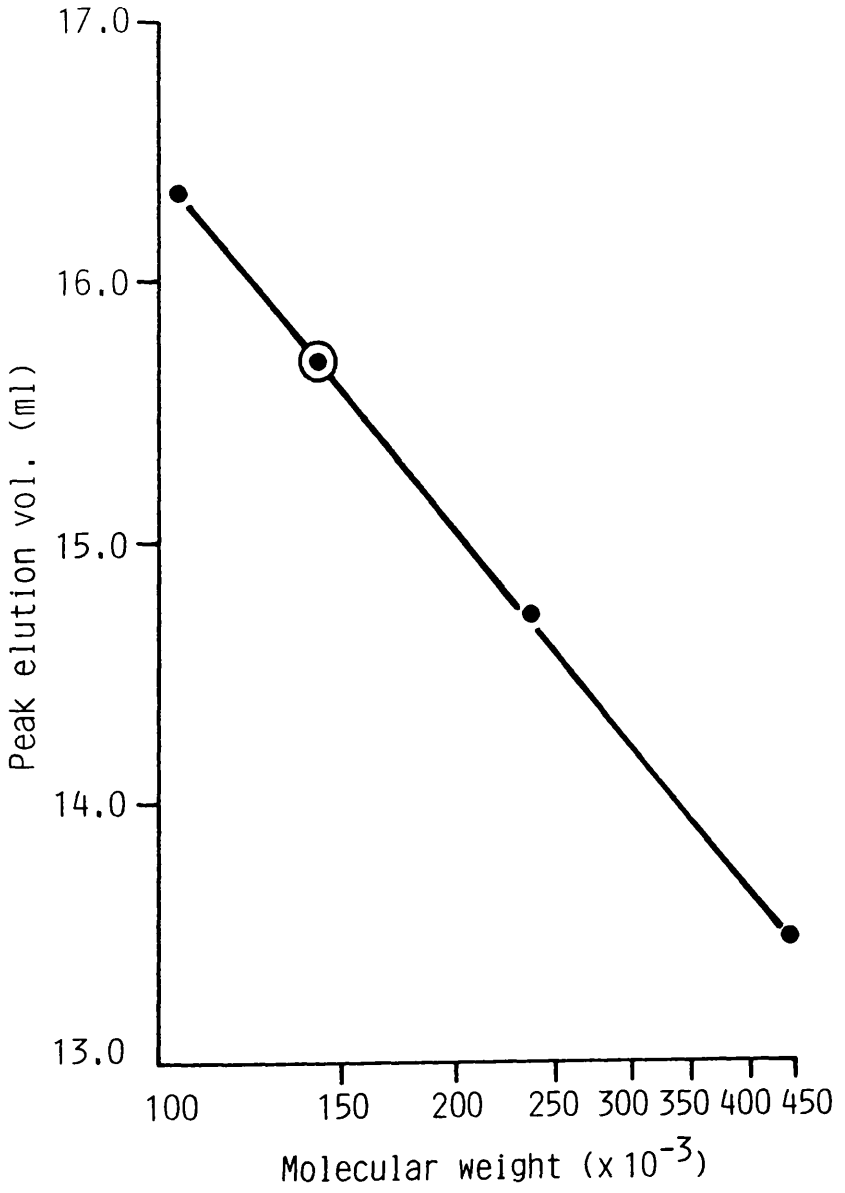


Figure 4.7 Superose 6 gel filtration standard curve

A Superose 6 gel filtration column was calibrated and run as described in Section 2.8.2(b). Peak elution volume was plotted against molecular weight. (•) standard proteins; (o) purified *E. coli* chorismate synthase. The standard curve was fitted by eye. NB. The column used in this experiment was not the same as that described in Section 3.7.

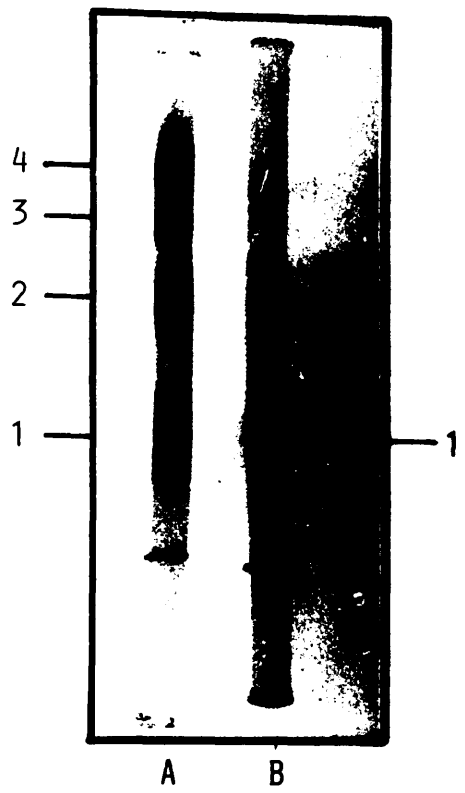


Figure 4.8 E. coli chorismate synthase crosslinked with dimethylsuberimidate

These 5% gels were run as described in Section 2.9 and stained for protein. Track A, 40ug of crosslinked E. coli chorismate synthase; track B, 8ug of E. coli chorismate synthase.

synthase activity. Using this assay procedure E. coli chorismate synthase was purified to homogeneity for the first time from the overexpressing strain. The enzyme required to be purified over 600 fold from crude extract and had a final specific activity of 14.8 units/mg. The purification procedure is outlined in Table 4.1 and a polyacrylamide gel showing the protein components at each stage is shown in Figure 4.5.

Three chromatographic steps were required to produce homogeneous enzyme. The first of these was anion-exchange chromatography on DEAE-Sephacel. Chorismate synthase activity eluted at a salt concentration of 180mM. The pooled active fractions from this step were then dialysed into a low phosphate buffer pH6.6 and subject to cation-exchange chromatography on cellulose phosphate. Phosphocellulose chromatography has shown to be a useful purification step for other characterised chorismate synthases (Cole & Gaertner, 1975., Boocock, 1983., Hasan & Nester, 1978). The enzyme was eluted from the column with a linear gradient of potassium phosphate. The material from this step showed only two protein components on SDS PAGE (Figure 4.5, track D). The final step in the purification procedure was hydrophobic interaction chromatography on Phenyl Sepharose. Chorismate synthase was eluted free from other contaminating polypeptides and showed only one band of protein of M_r 38 000 on SDS PAGE (Figure 4.5, track E). Purified E. coli chorismate synthase was stable for at least four months when stored in 50% glycerol at $-20^{o}C$.

Structural analysis of the purified enzyme from this study indicates that the E. coli chorismate synthase is a tetrameric protein of subunit M_r 38 000. This value agrees well with the

calculated subunit M_r of 38 183 deduced from the nucleotide sequence of the aroC gene and with the expression of a 38-40kDa polypeptide in an in vitro coupled transcription/translation of the plasmid pGM602 (Millar, 1986). Native M_r values obtained from f.p.l.c gel filtration studies agree with chemical crosslinking experiments and SDS PAGE. The E. coli chorismate synthase isolated in this study has a somewhat smaller subunit M_r than the bifunctional N. crassa enzyme (Table 3.4) and has no detectable diaphorase activity. The complete amino acid sequence has been deduced from the nucleotide sequence of the aroC gene (Millar, 1986) and confirmed by determining the N-terminal amino acid sequence of the purified enzyme (see Chapter 5).

CHAPTER 5 PRIMARY STRUCTURE ANALYSIS OF CHORISMATE SYNTHASE FROM E.COLI AND N.CRASSA

5.1 Introduction

The complete amino acid sequence of the E. coli chorismate synthase has been deduced from the nucleotide sequence of the aroC gene (Millar, 1986). Since no other chorismate synthase sequence has yet been reported comparisons at the level of primary structure were not possible. This chapter describes the determination of the N-terminal sequence of E. coli chorismate synthase and the deduction of the complete amino acid sequence for the gene sequence. Primary structure comparisons are made between the E. coli chorismate synthase and the partial sequences obtained from a proteolytic digest of the N. crassa polypeptide.

5.2 N-terminal sequence of the E. coli chorismate synthase

The N-terminal amino acid sequence of E. coli chorismate synthase was determined by automatic sequencing of the intact reduced and carboxymethylated protein as described in Materials and Methods. The yield of each residue recovered from each cycle of the sequencer and the derived sequence is shown in Figure 5.1. The N-terminal amino acid was found to be alanine indicating that the methionine corresponding to the start codon had been post-translationally removed. The first thirty amino acids were identified unambiguously and agreed exactly with the protein sequence predicted for the ORF from the DNA sequence (Figure 5.2). The repetitive yield from residues 1-30 was 93.6% by regression analysis.

Figure 5.1

The N-terminal amino acid sequence of the E. coli chorismate synthase

1 10

Ala-Gly-Asn-Thr-Ile-Gly-Gln-Leu-Phe-Arg-

11 20

Val-Thr-Thr-Phe-Gly-Glu-Ser-His-Gly-Leu-

21 30

Ala-Leu-Gly-Cys-Ile-Val-Asp-Gly-Val-Pro-

The sequence was determined on a liquid phase sequencer as described in Materials and Methods. The initial amount of protein sequencing was 30 nmol.

Sequence number	PTH-amino acid	nmoles recovered
1	Ala	15.8
2	Gly	10.6
3	Asn	9.4
4	Thr	2.9
5	Ile	3.5
6	Gly	8.9
7	Gln	11.8
8	Leu	8.5
9	Phe	6.4
10	Arg	n.d
11	Val	7.2

Sequence number	PTH-amino acid	nmoles recovered
12	Thr	4.0
13	Thr	3.6
14	Phe	5.7
15	Gly	6.0
16	Glu	6.3
17	Ser	1.8
18	His	3.5
19	Gly	4.7
20	Leu	2.5
21	Ala	4.5
22	Leu	2.4
23	Gly	3.8
24	Cys	0.5
25	Ile	2.0
26	Val	2.2
27	Asp	2.5
28	Gly	3.1
29	Val	2.4
30	Pro	1.4

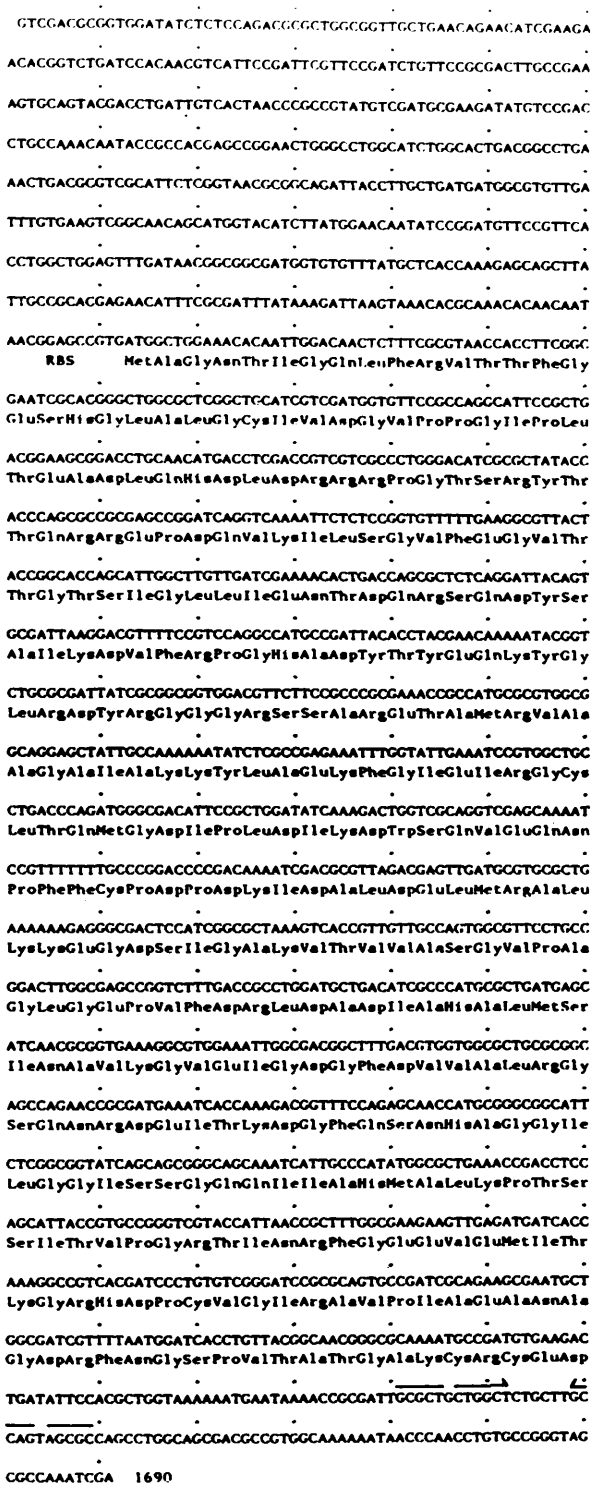


Figure 5.2 The E. coli aroC gene

The complete aroC coding sequence together with flanking 5' and 3' sequences is shown. The aroC structural gene and predicted amino acid sequence are indicated (positions 493-1562). The numbered amino acids in the aroC coding region are branched. The translational initiation codon is preceded by a proposed ribosome-binding site (RBS). The location of a potential inverted repeat structure is indicated by overlining. This sequence is taken from White et al. (1988).

5.3 Preparation of peptides from the N. crassa chorismate synthase

Direct N-terminal sequencing of the purified protein was unsuccessful. Amino acid sequence data was obtained by sequencing peptides generated from a complete clostripain digest of the reduced and carboxymethylated N. crassa chorismate synthase as described in Materials and Methods. Clostripain is a thiol protease which cleaves on the C-terminal side of arginyl residues and to a much lesser extent on the C-terminal side of lysyl residues. This secondary reaction can be reduced by carefully controlling the reaction time or eliminated by succinylation of the lysyl residues. Three peptides generated by this procedure were separated and sequenced.

5.3.1 HPLC separation of the peptides produced by a clostripain digest of the N. crassa chorismate synthase

The peptides resulting from the clostripain digest of the N. crassa chorismate synthase were separated by reverse phase chromatography on a Waters uBondapak C_{18} column. The material eluting from the column was monitored at 215nm and each peak collected by hand and labelled . Peaks that were discrete, abundant and had eluted in the later part of the gradient were selected for automatic gas-phase sequencing (Figure 5.3).

5.4 Sequencing of peptides separated by reverse phase H.P.L.C.

Four peptide peaks were selected from the h.p.l.c elution profile. Three of these peptides, labelled A, B and C on Figure 5.3, were sequenced on an automatic gas phase sequencer as described in Materials and Methods. A fourth peptide failed to be sequenced

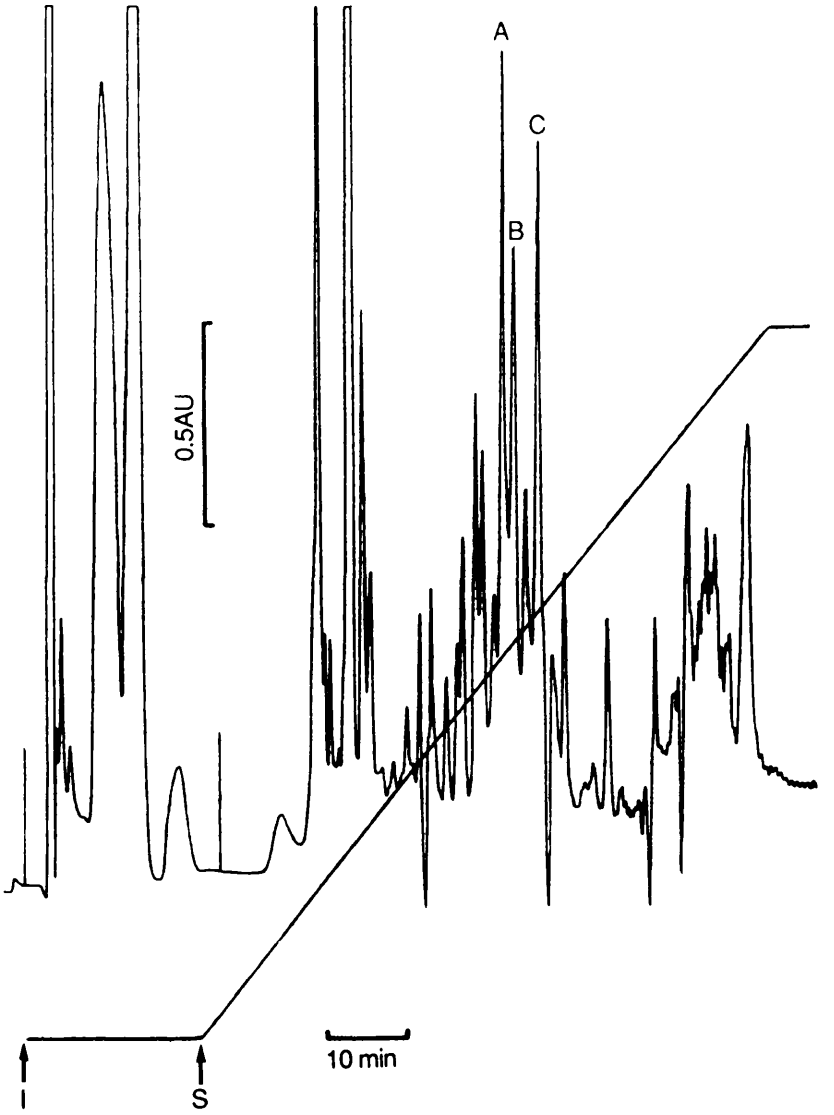


Figure 5.3 H.P.L.C. separation of the peptides produced by a clostripain digest of the N. crassa chorismate synthase

Conditions: Waters C_{18} reverse phase column. Solvent A, 0.1% TFA; solvent B, 0.05% TFA in methanol/acetonitrile/propan-2-ol (1/1/1); flow rate 1ml/min; gradient 0-70% B in 70 min.

I = Injection point
S = Start of gradient

and may have contained the blocked N-terminus. The yields of residues recovered from each cycle of the sequencer for each of the peptides are shown along with the derived amino acid sequences in Figure 5.4. In some cases two PTH-amino acid derivitives could be identified at the start of a sequence. This was due to small amounts of contaminating peptide and the PTH-amino acid derivitive of greatest abundance was used to derive the amino acid sequence. Sequencing continued until no further PTH-amino acid derivitives could be identified above background levels.

5.5 Comparison of the E. coli and N. crassa amino acid sequences

The sequences of the three peptides A, B and C derived from the proteolysis of the N. crassa chorismate synthase were aligned with the E. coli chorismate synthase sequence using the Bestfit sequence comparison programme of the University of Wisconsin Genetics Computer Group. Each peptide shows homology with a region of the E. coli polypeptide (Figure 5.5).

5.6 Discussion

In order to confirm the location of the correct open reading frame for the DNA sequence of E. coli chorismate synthase the purified enzyme was subject to direct N-terminal amino acid sequencing. The predicted amino acid sequence exactly matched the first thirty amino acids derived from N-terminal sequencing and the complete amino acid sequence could then be deduced (Figure 5.2). To compare the primary structures of both the E. coli and N.crassa enzymes it was necessary to sequence the N. crassa polypeptide directly. Direct sequencing from the N-terminal amino acid was

unsuccessful. Therefore a complete clostripain digest of the chorismate synthase polypeptide was performed. Clostripain (clostridiopeptidase B, E.C. 3.4.4.20) is a sulphydryl proteolytic enzyme which is obtained from the culture filtrate of Clostridium histolyticum. The enzyme possesses a protease activity with a highly limited specificity directed at the carboxyl linkage of arginyl residues (Mitchell & Harrington, 1971). To minimise this secondary reaction the incubation time and reaction temperature were controlled carefully. The unique specificity of the enzyme was used to generate large initial peptide fragments suitable for sequencing. Three peptides labelled A,B and C were isolated by reverse phase h.p.l.c and sequenced. The peptides varied in length from 19 to 26 residues and the occurrence of a lysine in peptide A indicated that proteolysis at this site was not a significant problem. All three peptides show degrees of homology with stretches of the E. coli sequence. These regions of sequence homology occur toward the C-terminus of the E. coli enzyme.

5.7 Summary

The complete amino acid sequence of the E. coli chorismate synthase (the aroC gene product) has been determined by a combined nucleotide and direct amino acid sequencing strategy. Direct amino acid sequencing of peptide fragments of the N.crassa chorismate synthase has revealed stretches of significant homology with the E. coli sequence.

Figure 5.4

The amino acid sequence of peptides A,B and C

Peptide A:

1

Asn/Ser-Val/Leu-Leu-Ser/Ala-Gly/Ala-Leu-Lys-Gln/Tyr-

10

Thr-Ile/Ala-Asn-Ser-Gly-Lys-Asp-Thr-Val-Gly-Asn-

20

Ile-

Peptide B:

1 10

Thr-Asn-Phe-Ser-Gly-Gly-Ile-Gln-Gly-Gly

Ile-Ser-Asn-Gly-Ala-(-)-Ile-Tyr-Phe-

Peptide C:

1

Ser/Val/Leu-Phe/Val/Glu-Glu-Val-Gly-Ser-Gly-Phe-

10

Gly-Gly-(-)-Glu-Val-(-)-Gly-Ser-Ile-His-Asn-

20

Glu-Gln-Phe-Val-(-)-Ala-Glu

The sequences were determined on a gas phase sequencer as described in Materials and Methods. The presence of contaminating peptide is reflected in the occurrence of several amino acids at the start of sequences A and C.

Figure 5.4 (continued)

Peptide A

Sequence number	PTH-amino acid	nmoles recovered
1	Asn+Ser	0.24+0.4
2	Val+Leu	0.32+0.34
3	Leu	0.7
4	Ser+Ala	n.d.+0.08
5	Gly+Ala	0.15+0.08
6	Leu	0.7
7	Lys	0.25
8	Gln+Tyr	0.55+0.09
9	Thr	n.d.
10	Ile+Ala	0.23+0.03
11	Asn	0.22
12	Ser	0.04
13	Gly	0.12
14	Lys	0.08
15	Asp	0.12
16	Thr	0.02
17	Val	0.11
18	Gly	0.10
19	Asn	0.08
20	Ile	0.05

Peptide B

Sequence number	PTH-amino acid	nmoles recovered
1	Thr	0.25
2	Asn	0.36
3	Phe	0.41
4	Ser	0.09
5	Gly	0.40
6	Gly	0.43
7	Ile	0.38
8	Gln	0.42
9	Gly	0.39
10	Gly	0.40
11	Ile	0.37
12	Ser	0.18
13	Asn	0.18
14	Gly	0.17
15	Ala	0.18
16	-	-
17	Ile	0.20
18	Tyr	0.14
19	Phe	0.15

Peptide C

Sequence number	PTH-amino acid	nmoles recovered
1	Ser+Val+Leu	0.05+0.09+0.12
2	Phe+Val+Glu	0.8+0.03+0.08
3	Glu	0.77
4	Val	0.70
5	Gly	0.45
6	Ser	0.18
7	Gly	0.30
8	Phe	0.40
9	Gly	0.40
10	Gly	0.49
11	-	-
12	Glu	0.40
13	Val	0.4
14	-	-
15	Gly	0.19
16	Ser	0.09
17	Ile	0.21
18	His	0.07
19	Asn	0.10
20	Glu	0.08
21	Gln	n.d.
22	Phe	n.d.
23	Val	0.05
24	-	-
25	Ala	0.02
26	Glu	-

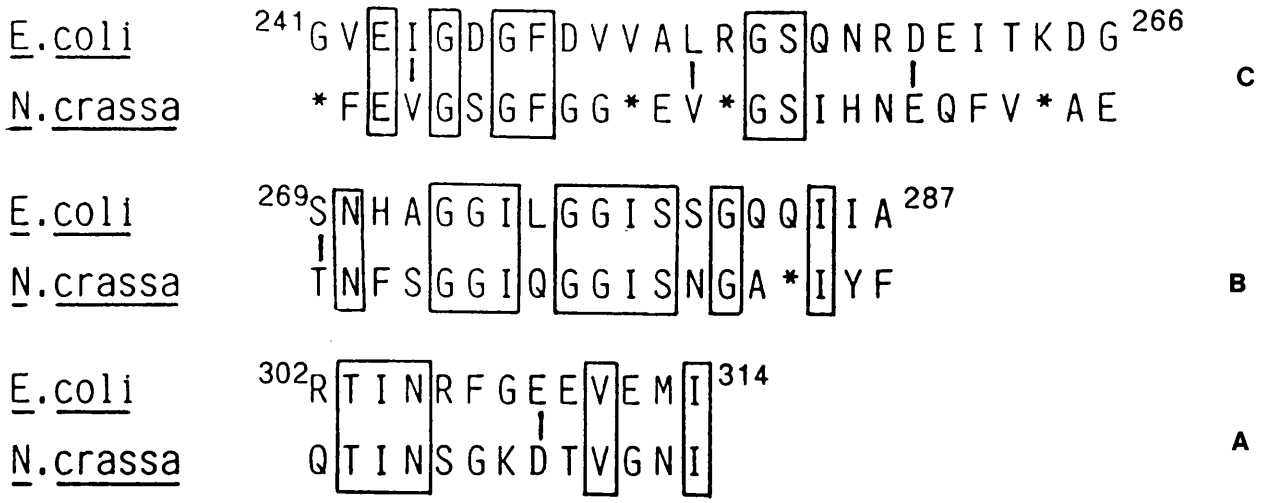


Figure 5.5 The alignment of peptides A,B and C with the sequence of the E. coli chorismate synthase.
The sequences were aligned using the Bestfit sequence comparison program of the University of Wisconsin Genetics Computer Group. Identical amino acids are boxed; a conservative substitution is represented by a vertical line and an asterisk indicates a residue that could not be unambiguously identified.

CHAPTER 6 FLAVIN AND THE E. COLI CHORISMATE SYNTHASE

6.1. Introduction

All chorismate synthases studied to date show an absolute requirement for a reduced flavin cofactor. The catalytic relationship between reduced flavin and the synthesis of chorismic acid is not clear. Concentrated samples of the purified *E. coli* and *N. crassa* chorismate synthases (2mg/ml) from this study were not coloured. Spectrophotometric analysis of the *E. coli* enzyme (1mg/ml solutions) did not reveal any strong absorption peaks in the visible region of the spectrum (300-600nm). This preliminary evidence suggested that although both enzymes required flavin for enzymic activity neither protein retained significant amounts of the cofactor throughout purification.

This chapter gives details of experiments investigating further the relationship between the flavin cofactor and *E. coli* chorismate synthase. Spectrophotometric fluorometric and kinetic analyses are presented and a possible flavin binding domain is identified from the *E. coli* amino acid sequence.

6.2. Further overexpression of the E. coli chorismate synthase

To facilitate easier purification of the *E. coli* chorismate synthase the *E. coli* *aroC* gene was subcloned further to allow IPTG-induced elevation of *aroC* expression in *tac-aroC* constructs (Millar, 1986). *E. coli* AB2849/pGM605 cells were grown on a preparative scale following induction with IPTG (see Materials and Methods).

Chorismate synthase activity in crude extracts of these cells indicated a level of overexpression nearly 500-fold that of similar E. coli K12 preprations. Purification of the enzyme from this overexpressing strain was attempted and is outlined in the following section.

6.2.1 Purification of chorismate synthase from E. coli AB2849/pGM605

The purification of chorismate synthase from this strain followed exactly the scheme described in Chapter 4. 20g of wet weight E. coli AB2849/pGM605 cells were suspended in 10ml of extraction buffer and passed twice through an automatic French Pressure cell. Cell debris and other insoluble material were removed by centrifugation and the supernatant subjected to ammonium sulphate fractionation. Three chromatographic steps involving anion-exchange, cation-exchange and hydrophobic interaction chromatography were then used to yield pure enzyme. The purification procedure is outlined in Table 6.1. Enzyme purified from this source had a subunit M_r of 38 000 by SDS PAGE and a specific activity similar to chorismate synthase purified from AB2849/pGM602 cells.

6.2.2 Purification problems

Although the expresssion of chorismate synthase in E. coli AB2849/pGM605 was in vast excess of E. coli K12 'wild-type' levels and much greater than that in cells containing the plasmid pGM602, overall yield of the enzyme after purification was very low (Table 6.1). Whilst monitoring the purification procedure it was noted that large losses of chorismate synthase activity were occurring. The greatest losses were associated with the precipitation of substantial amounts of protein during dialysis after ammonium

Table 6.1

Purification of chorismate synthase from *E. coli*

The results are for a purification starting from 19g of *E. coli* AB2849/pGN605 cells

Step	Volume (ml)	Con^{cn} of protein (mg/ml)	Activity (units/ml)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold	Yield (%)
A Crude extract	66	18.1	73.6	4858	4.1	1	100
B 35-60% $AmSo_4$	96	10.3	29.1	2794	2.8	0.7	58
C DEAE-Sephacel	44	3.6	41.0	1804	11.4	2.8	37
D P_{11}	34	0.45	7.1	242	15.8	3.8	5.0
E Phenyl Sepharose	22	0.31	11.0	243	35.2	8.6	5.0

sulphate fractionation and prior to phosphocellulose chromatography. This precipitate contained significant levels of chorismate synthase activity. Another more surprising and visible feature of this purification was the deep yellow colour of the crude extract and the soluble material after ammonium sulphate precipitation. Dialysis allowed this yellow chromophore to be collected and analysed further.

6.3 Isolation and identification of the dialysable cofactor

The strong yellow colour of the chromophore collected in the dialysis medium during the purification of chorismate synthase from AB2849/pGM605 cells suggested strongly the presence of high concentrations of flavin. In an attempt to characterise this component further a series of spectrophotometric, fluorometric and enzyme analyses were performed and are described in the following sections.

6.3.1 Spectrophotometric analysis

An aliquot of the dialysis buffer containing the chromophore was removed and freeze-dried to concentrate the sample. The freeze-dried solid was resuspended in 1ml of distilled water to give a solution with a strong yellow colour. The visible absorption spectrum of this solution was determined. Maxima were obtained at 350 and 430nm. The solution could be bleached by the addition of 1mM sodium dithionite, with the loss of the absorption peaks (Figure 6.1).

6.3.2 Fluorometric analysis

Chromophore diluted in 50mM-potassium phosphate buffer pH7.0 showed characteristic flavin excitation and emission spectra

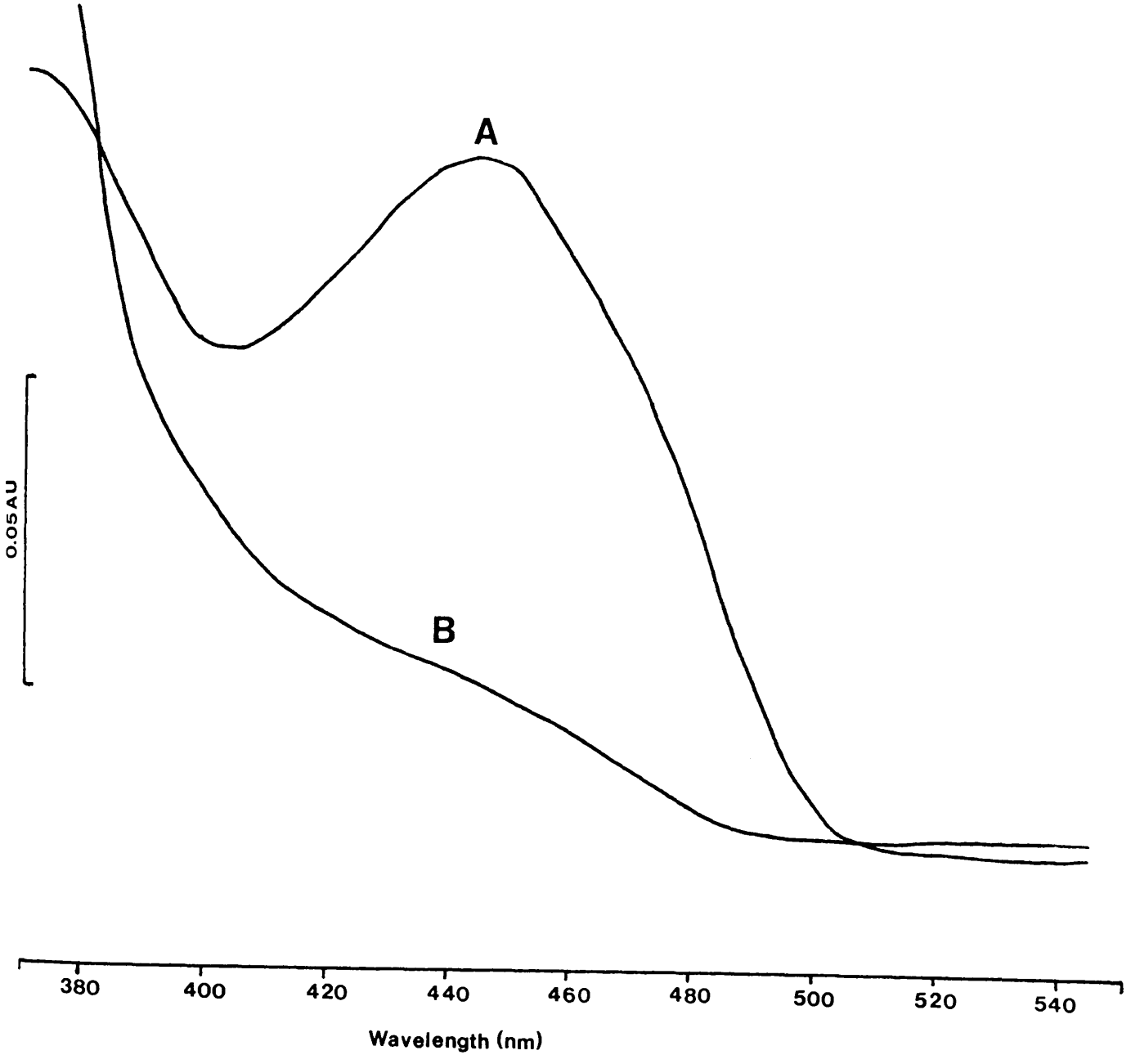


Figure 6.1 The absorption spectrum of the chromophore overproduced by E. coli AB2849/pGM605

The spectrum was determined using a double beam spectrophotometer as described in Section 2.10. Line A, absorption spectrum; line B, absorption spectrum upon reduction with dithionite.

with maxima at 380 and 448nm (Excitation spectrum) and at 525nm (Emission spectrum) (Figure 6.2).

6.3.3 Flavin composition

The absorption and fluorometric spectra of the unknown chromophore suggested it was indeed a flavin type molecule. Since the excitation and emission spectra of FMN, FAD and riboflavin are generally identical within experimental error further chemical modification and fluorometric analysis was required to determine the flavin composition of the solution. Under controlled conditions the labile phosphoanhydrate bond of FAD may be hydrolysed converting it to FMN (Koziol, 1971) and since FMN has a fluorescence nearly 10-fold greater than FAD any increase in fluorescence after hydrolysis may be attributed to FAD. Aliquots of the solution under investigation were removed and treated with 0.1N HCl on ice and at 100^{o}C for 15 min (standard control solutions of FAD and FMN were treated similarly). The acid solutions were then neutralised with an equivalent volume of NaOH, diluted with 50mM-potassium phosphate buffer pH7.0 to a final volume of 3ml and measured for fluorescence. There was no significant change in fluorescence of the unknown sample after acid hydrolysis at 100^{o}C (Table 6.2).

6.3.4 Enzymatic analysis

At concentrations equivalent to 10uM-FMN the chromophore was able to maximally stimulate *N. crassa* chorismate synthase activity (aerobic assays) and *E. coli* chorismate synthase activity (anaerobic assays) when added to assay cocktails lacking this cofactor.

Table 6.2 Flavin composition of the unknown chromophore

Final concentration of standard flavins (FMN, FAD) prior to fluorometric analysis = 0.1uM

Sample	Control (No treatment)	relative fluorescence (control=100) acid treatment ($0^{o}C$)	acid treatment ($100^{o}C$)
FMN	100	98.4	97.6
FAD	100	98.8	7.33
unknown	100	97.1	94.2

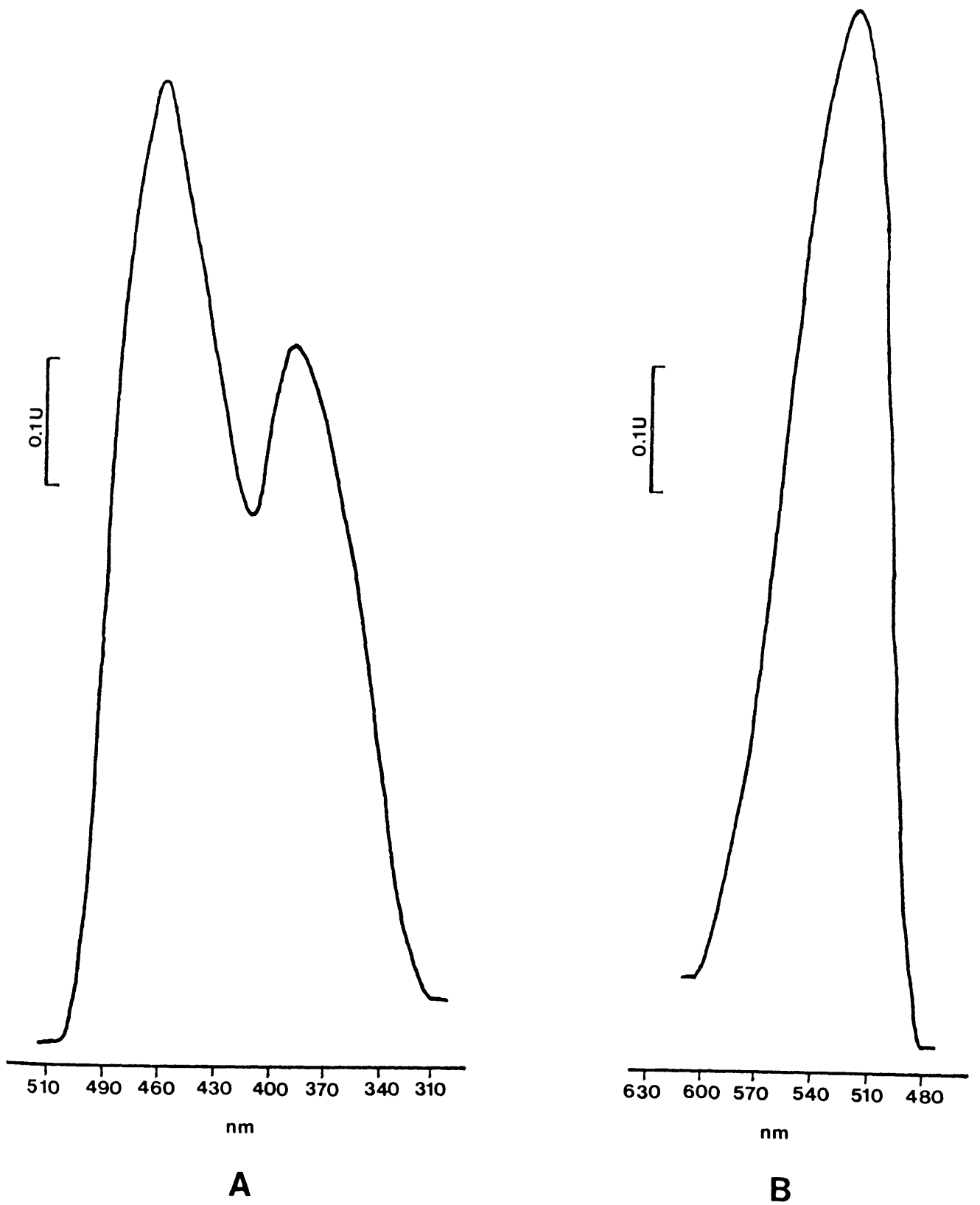


Figure 6.2 The excitation and emission spectra of the overproduced chromophore

Excitation and emission spectra were determined by fluorescence spectroscopy as described in Section 2.10. A, excitation spectrum; B, emission spectrum.

6.4 Fluorometric analysis of the E. coli chorismate synthase

The fluorescence spectrum of the E. coli chorismate synthase was studied. Solutions of the enzyme (0.5mg/ml in 50mM-potassium phosphate buffer pH7.0) showed weak excitation and emission spectra characteristic of bound flavin. Maxima were obtained at 374 and 446nm (Excitation spectrum) and at 528nm (Emission spectrum). The excitation and emission spectra of E. coli chorismate synthase and that of FMN are compared in Figure 6.3.

6.5. Preference of the E. coli chorismate synthase for FMN as the flavin cofactor

E. coli chorismate synthase was assayed anaerobically as described in Chapter 4 except that enzyme activity was monitored using each of the three flavin cofactors; FMN, FAD and riboflvain (Table 6.3). Chorismate synthase activity measured in the presence of FAD was only 48% of that monitored using FMN. Riboflavin was the least preferred substrate and the enzyme showed less than 10% of the activity recorded in the presence of FMN.

6.6 Identification of a possible flavin binding region

Fluorometric analysis of the purified E. coli chorismate synthase indicated that limited amounts of flavin remained bound to the protein molecule throughout the purification procedure. The interaction of flavins with particular stretches of polypeptide chain and to specific amino acid side chains has been documented for a number of FAD and FMN binding proteins (Mayhew & Ludwig, 1975; Wood et al., 1984; Porter & Kasper, 1986). The availability of

Figure 6.3 The flavin fluorescence spectrum of the *E. coli* chorismate synthase

The flavin emission and excitation spectra were determined by fluorescence spectroscopy as described in Section 2.10.

A, flavin excitation spectrum
B, flavin emission spectrum
C, excitation spectrum of FMN (1uM)
D, emission spectrum of FMN (1uM)

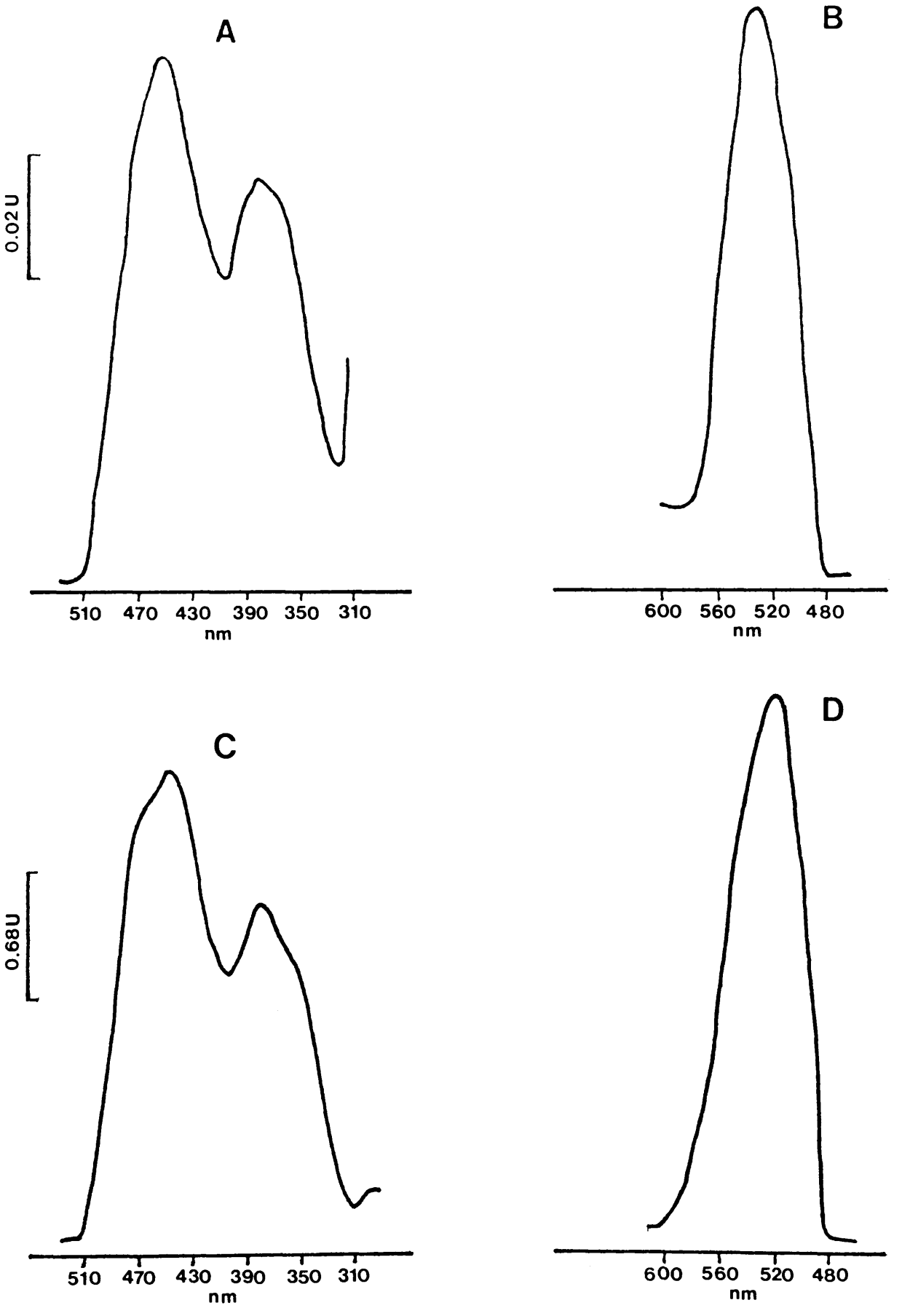

A
0.02U
510 470 430 390 350 310
nm
B
600 560 520 480
nm
C
0.68U
510 470 430 390 350 310
nm
D
600 560 520 480
nm

Table 6.3 Preference of E. coli chorismate synthase for FMN as a cofactor

Flavin cofactor	nmoles chorismate formed /5min
FMN (10uM)	0.82
FAD (10uM)	0.39
Riboflavin (10uM)	0.06

The results represent an average of three assays over 5 minutes for each flavin cofactor. Results did not differ by more than 5%. Chorismate synthase activity was monitored anaerobically as described in Chapter 2. Each assay contained 8mU of highly purified E. coli chorismate synthase.

the complete amino acid sequence for the E. coli chorismate synthase permitted a comparison of this enzyme with other flavin binding proteins.

Enzymes that bind FAD exhibit a common secondary structural motif, the FAD-binding fold (Thieme et al., 1981, Wierenga et al., 1983) and a high degree of sequence homology within the β-α-β segment that contains the AMP-binding site (Rice et al., 1984). Analysis of the E. coli chorismate synthase sequence did not reveal any significant sequence homology with a variety of FAD flavoproteins. FMN binding proteins have been characterised less extensively although detailed three dimensional structures of the flavodoxins from Desulfovibrio vulgaris and Clostridium pasteurianum are known along with the corresponding amino acid sequences (Watenpaugh et al., 1973; Burnett et al., 1974; Tanaka et al., 1974). The amino terminal portions of these enzymes were found to be important in binding directly the FMN prosthetic group and showed sequence homology with other bacterial flavodoxins as well as the FMN binding domain of NADPH-cytochrome P-450 oxidoreductase from rat liver (Porter & Kasper, 1986). Residues 70-81 of the E. coli chorismate synthase show limited homology with this consensus FMN binding region (Figure 6.4).

6.7 Discussion

Gross over-expression of the E. coli chorismate synthase polypeptide in tac-aroC constructs resulted in the concommitant production of large amounts of a yellow chromophore. The chromophore could be isolated free from protein by dialysis.

Evidence that the chromophore was a flavin type molecule was obtained from spectrophotometric and fluor ometric analysis. Visible absorption maxima at 350 and 430nm compare well with those documented for FMN and FAD. The ability to bleach the chromophore by reduction and the loss of the absorption maxima is a feature common to flavins. Fluorometric analysis showed excitation and emission spectra characteristic of flavins. Having established the flavin character of the chromophore, further analysis was needed to identify the molecule as either FMN or FAD. Acid hydrolysis at 100^{o}C did not result in any increase in fluorescence as would have been expected if the solution contained FAD or a mixture of FAD and FMN. Finally, the ability of the chromphore to maximally stimulate N. crassa chorismate synthase activity (a FMN specific enzyme, Boocock, 1983) identified it as almost certainly FMN.

Although highly purified extracts of the E. coli chorismate synthase showed no colour and did not have any visible absorption maxima, fluorometric analysis of the enzyme showed weak excitation and emission spectra characteristic of bound flavin. The paucity of bound cofactor was reflected in that concentrations of enzyme (13uM) showed roughly 450 fold less fluoroscence than similar concentrations of free FMN and that enzyme activity could not be detected in the absence of exogenous FMN in anaerobic assays of up to 15 minutes duration. These results suggest that although chorismate synthase is a flavoenzyme the flavin is loosely associated with the protein and is almost completely lost during purification.

Anaerobic assays showed the enzyme to prefer FMN as a flavin substrate over FAD and riboflavin. Enzyme activity in the

presence of FMN was twice that in the presence of FAD. The absolute requirement for flavin and preference for FMN suggested there might be a region of the polypeptide chain with sequence homology to other FMN binding flavoproteins. Comparison by eye with the sequence of the flavodoxin D. vulgaris and rat liver P-450 oxidoreductase revealed some homology with the proposed FMN phosphate binding regions of these proteins. D. vulgaris flavodoxin and P-450 oxidoreductase have a region of polypeptide chain near their N-termini containing a cluster of serine and threonine residues which hydrogen bond the FMN phosphate oxygens through their side-chain hydroxyl groups (Watenpaugh et al., 1973). The E. coli chorismate synthase sequence shows a similar cluster of serine and threonine residues towards the N-terminus (residues 71-80).

The results from these experiments highlight the relationship between the E. coli chorismate synthase and flavin. Parallel overexpression of FMN is seen in response to the expression of high levels of chorismate synthase. The flavin is not bound strongly to protein and can be isolated by dialysis. The purified enzyme contains limited amounts of flavin, detectable only by fluorescence spectroscopy and requires the addition of exogenous FMN for maximal activity. High concentrations of FMN in the extracts created problems during the purification and it seems likely that the flavin was the cause of precipitation of the enzyme. The evidence for the association between chorismate synthase and FMN was strengthened further by the identification of a possible FMN-binding sequence near the N-terminus of the enzyme.

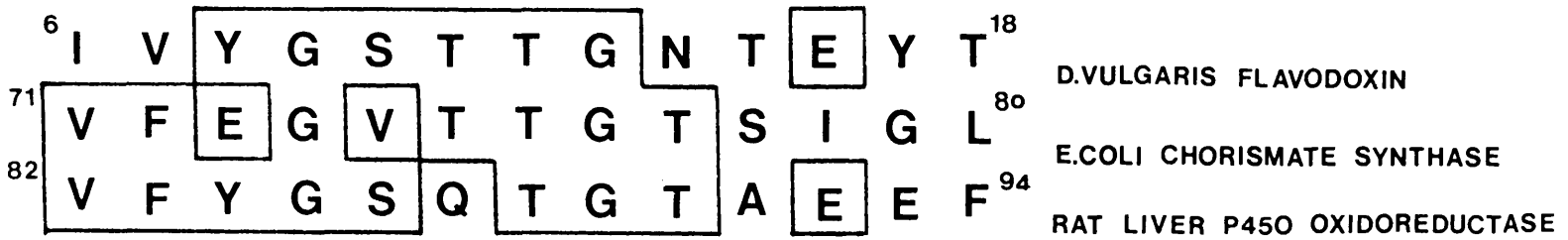


Figure 6.4 A putative flavin binding region in the E. coli chorismate synthase

Alignment of the E. coli chorismate synthase sequence with the FMN binding region of D. vulgaris flavodoxin and rat liver P_{450} oxidoreductase. The sequences were aligned by eye and identical amino acids are boxed.

6.8 Summary

E. coli cells containing the plasmid pGM605 produced high levels of FMN in response to IPTG-induced elevation of *aroC* expression. Chorismate synthase purified from this source contained limited amounts of bound flavin and could be maximally stimulated by FMN in anaerobic assays. A possible FMN binding domain is identified from the *E. coli* sequence.

CHAPTER 7 THE REACTION MECHANISM OF CHORISMATE SYNTHASE

7.1. Introduction

The reaction mechanism of chorismate synthase is poorly understood. The synthesis of chorismic acid from EPSP does not involve a net redox change and therefore the requirement for FMN and NADPH is wholly mysterious. A number of hypotheses have been proposed in an attempt to explain the unusual stereochemistry of the reaction and the necessity for a reduced flavin cofactor (see Chapter 1). This chapter sets out to define the minimum conditions necessary for activity in both the E. coli and N.crassa enzymes and highlights differences and similarities in catalytic behaviour between these two systems.

7.2. Cofactors and assay conditions suitable for chorismate synthase activity

Early work on the N.crassa and E. coli chorismate synthases showed both enzymes to be active under a wide and sometimes contradictory set of assay protocols (Morell et al., 1967; Welch et al; 1974 Boocock, 1983). Morell et al. claimed the E. coli enzyme could be activated by a variety of 'reducing' conditions the most interesting of which was the observation that enzyme activity could be detected in the presence of reductant (NADH or dithionite) but no exogenous flavin. This activity was seen only after protracted lag periods (15 min) and was almost certainly due to the presence of catalytic amounts of flavin in the rather crude extracts under study. Significant lags could be eliminated if the enzyme was assayed in the presence of both reductant and exogenous flavin in an O_2 free environment. Purified preparations of the E. coli

chorismate synthase from this study show an absolute requirement for a reduced flavin cofactor which can be generated either chemically *via* dithionite or enzymically in a coupled assay with excess exogenous diaphorase (pig heart lipoamide dehydrogenase). The *N.crassa* chorismate synthase appears to exist as a bifunctional diaphorase/chorismate synthase unit (Boocock, 1983) (Chapter 3) and as such has the ability to generate catalytic amounts of reduced flavin intrinsically. The enzyme can be assayed under both aerobic and anaerobic conditions and shows maximal activity in anaerobic assays with chemically generated $FMNH_2$ or $FADH_2$ (Table 7.1). Physiologically FMN and NADPH are the preferred substrates for the diaphorase activity (Boocock, 1983) and *in vivo* $FMNH_2$ is almost certainly the cofactor necessary for chorismate synthesis. Assay conditions found to be suitable for the detection of chorismate synthase activity in this study are outlined in Table 7.2. Both chorismate synthases require reduced flavin for activity, and although either $FMNH_2$ or $FADH_2$ can activate the enzymes when supplied directly, $FMNH_2$ appears to be the preferred cofactor.

7.3 The activation of the N.crassa chorismate synthase

The ability to assay the *N.crassa* enzyme continuously under aerobic conditions has allowed much preliminary kinetic analysis to be undertaken (Boocock, 1983). Unusually the enzyme shows lags in chorismate synthesis. The addition of enzyme to a complete assay cocktail results in a significant lag before a final linear rate is achieved. Pre-incubation with FMN+EPSP but NOT FMN+NADPH or EPSP+NADPH results in a substantial reduction but not a complete elimination of this lag period. Boocock suggested that these lags were a result of slow conformational changes in the enzyme

Table 7.1

Activation of N. crassa chorismate synthase with chemically and enzymically reduced flavins.

Concentrations of cofactors (all assays contained 50uM-EPSP)	Relative chorismate synthase activity
20uM NADPH (aerobic)	
+ 10uM FMN	100
+ 10uM FAD	20 (after 10 min)
2mM dithionite (anaerobic)	
+ 10uM FMN	318
+ 10uM FAD	188

12mU of highly purified N. crassa chorismate synthase was assayed aerobically and anaerobically in 50mM-potassium phosphate buffer pH 7.0. The aerobic assays were monitored continuously at 275nm. The progress of the anaerobic assays was monitored using the stopped time method described in Chapter 4.

Table 7.2

Cofactors and assay conditions suitable for chorismate synthase activity.

	Concentrations of cofactors (all assays contained 50uM EPSP)	chorismate synthase activity *E. coli*	*N. crassa*
A	10uM FAD 20uM NADPH	no	trace (see Table 7.1)
B	10uM FAD 20uM NADH	no	no
C	10uM FMN 20uM NADH	no	no
D	10uM FMN 20uM NADPH	no	yes (see Table 7.1)
E	2mM dithionite 20uM NADPH 20uM NADH	no	no
F	2mM dithionite 10uM FAD	yes (see Table 6.3)	yes (see Table 7.1)
G	2mM dithionite 10uM FMN	yes (see Table 6.3)	yes (see Table 7.1)
H	Pig heart diaphorase 10uM FAD 50uM NADH	yes	no data

The assay conditions for each of the experiments A-G were as follows: assays A,B,C and D; enzyme (12mU of highly purified *E. coli* or *N. crassa* chorismate synthase) was aerobically assayed in 50mM-TEA/Cl KOH buffer pH 7.0, containing 50mM-KCl and the relevant cofactors. The final reaction volume was 1ml and the progress of the chorismate synthase reaction was followed at 275nm using a Gilford Spectrophotometer. Assays E, F and G; enzyme (12mU of highly purified *E. coli* or *N. crassa* chorismate synthase) was assayed in 50mM-potassium phosphate buffer pH 7.0, containing 2mM dithionite and the relevant cofactors. The final reaction volume was 1ml and the progress of the chorismate synthase reaction was followed using the stopped time h.p.l.c method described in Chapter 4.

Assay H; enzyme (12mU of highly purified *E. coli* chorismate synthase) was assayed in 50mM-TEA/Cl KOH buffer pH 7.0, containing 50mM KCl, 10mM L-glutamine, 5mM $MgCl_2$, 50mU of pig heart diaphorase and sufficient partially purified *N. crassa* anthranilate synthase (free from any contaminating chorismate synthase activity) to observe a rate of anthranilate production. The final assay volume was 3ml and the progress of the reaction was followed by monitoring anthranilate production fluorometrically (excitation/emission wavelengths of 315/380nm). This experiment was of qualitative value only, as insufficient *N. crassa* anthranilate synthase was available to enable complete coupling of the two reactions.

associated with the binding of FMN and EPSP, 'locking' FMN into it's binding site. This hypothesis is supported by earlier work (Welch et al., 1974) where enzyme pre-incubated with FMN+EPSP and then gel filtered was found to show marked activity without lags when assayed in the absence of FMN. The ability of the N.crassa enzyme in this study to utilise $FMNH_2$ directly suggests that the conformational change and tight binding of FMN is a necessary pre-requisite for reduction of the flavin moiety and the maintenance of this reduced state in a potentially oxidising environment.

7.4. Fluorescence studies and the binding of EPSP to the E. coli chorismate synthase

The combination of a stopped assay and a requirement for strict reducing conditions has made a kinetic analysis difficult using conventional apparatus. Problems in measuring the initial rate of reaction during the first minutes of catalysis precludes the ability to measure lags in enzyme activation. In an attempt to detect any possible conformational changes of the E. coli chorismate synthase in the presence of substrates, a fluorometric study of the enzyme was undertaken. Fluorescence spectroscopy is a useful tool in following enzyme conformation (Friefelder, 1986). The E. coli chorismate synthase contains two natural fluors which can be studied exclusively; limited amounts of bound flavin and a single tryptophan residue at position 172 in the polypeptide chain.

7.4.1 Flavin fluorescence

The weak flavin fluorescence of the highly purified E. coli chorismate synthase is described in detail in Chapter 4. The response of the Em and Ex spectra of the enzyme bound flavin upon

addition of substrate was investigated (Figure 7.1). Both fluorescence spectra show significant quenching in the presence of EPSP. Quenching takes the form of a slow decrease in fluorescence reaching 50% of maximum fluorescence after 16 min (Figure 7.2).

7.4.2 Aromatic fluorescence

The E. coli chorismate synthase shows a characteristic tryptophan Em spectrum (λ_m= 340nm) when excited at 280nm (Figure 7.3). This intrinsic fluor was used to study conformational changes of the enzyme in the presence of substrates and cofactors. The addition of either FMN or FAD to the enzyme resulted in a rapid ($t < 3s$) quenching of the Em spectrum, suggesting that the flavin was binding at or near the tryptophan residue. Addition of EPSP to the enzyme-FMN complex gave a further 'slow' quenching which was complete in 10 min. EPSP + enzyme-FAD did not show a similar 'slow phase' reduction in fluorescence, Figure 7.4. The interaction between the E. coli enzyme and the substrates FMN and EPSP appears to involve a biphasic tryptophan quenching response. Flavin, either FAD or FMN can bind to the enzyme, presumably at the flavin binding site causing a 50% decrease in fluorescence, EPSP then binds to this complex causing a conformational change that results in further quenching. Pre-incubation with EPSP was not found to decrease the time required for the slow phase quenching.

7.4.3 Substrate binding models

The results described in the above sections are consistent with the slow activation model proposed by Boocock for the N.crassa chorismate synthase. The enzyme reversibly binds FMN which is then 'locked' into its binding site by a further EPSP-enzyme

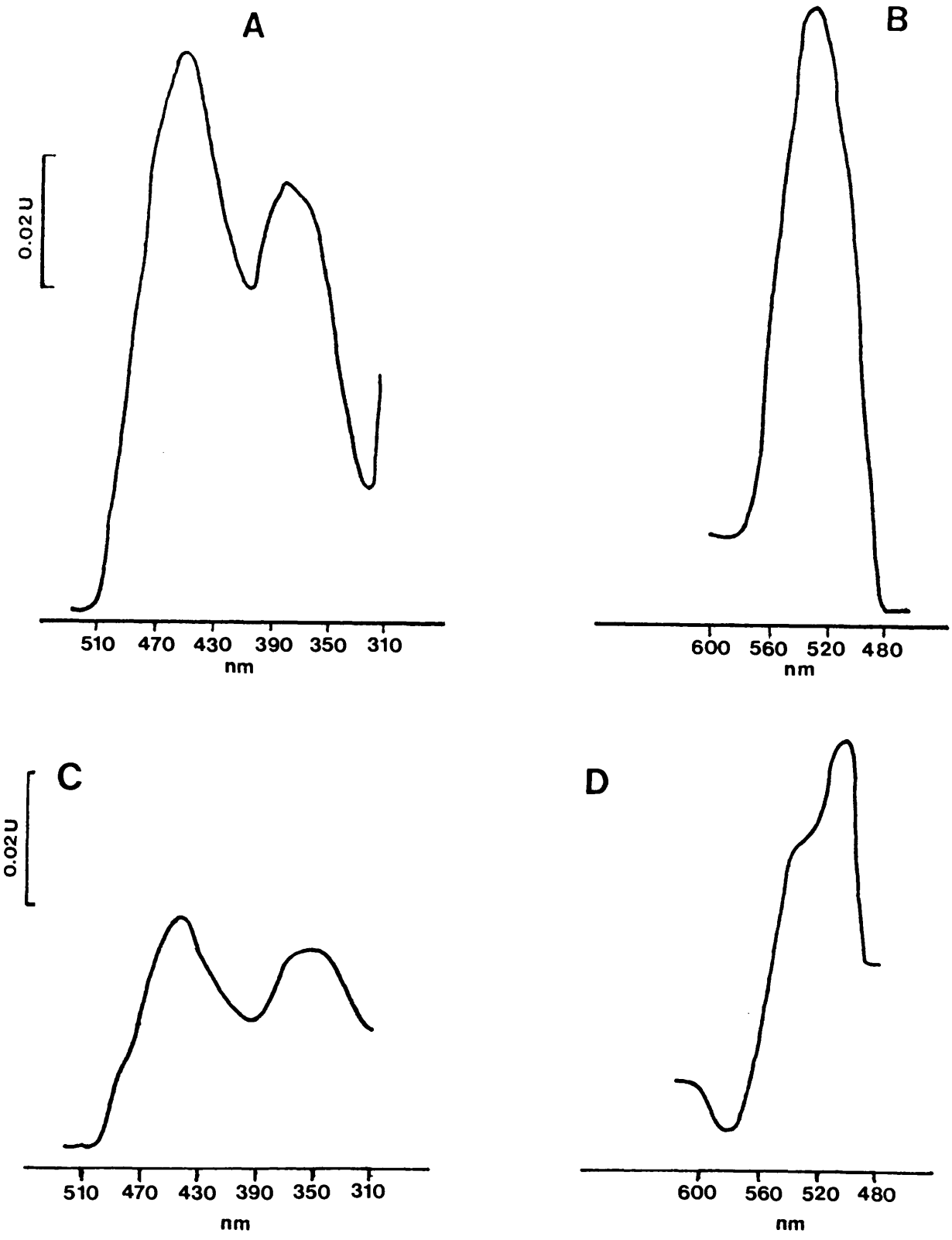


Figure 7.1 The quenching of flavin fluorescence with EPSP

Solutions of E. coli chorismate synthase, 0.5mg/ml in 50mM-potassium phosphate buffer pH7.0, were subjected to fluorescence spectroscopy as described in Section 2.10

A, excitation spectrum
B, emission spectrum
C and D, excitation and emission spectra after incubation with EPSP (final concentration 40uM) for 16 min.

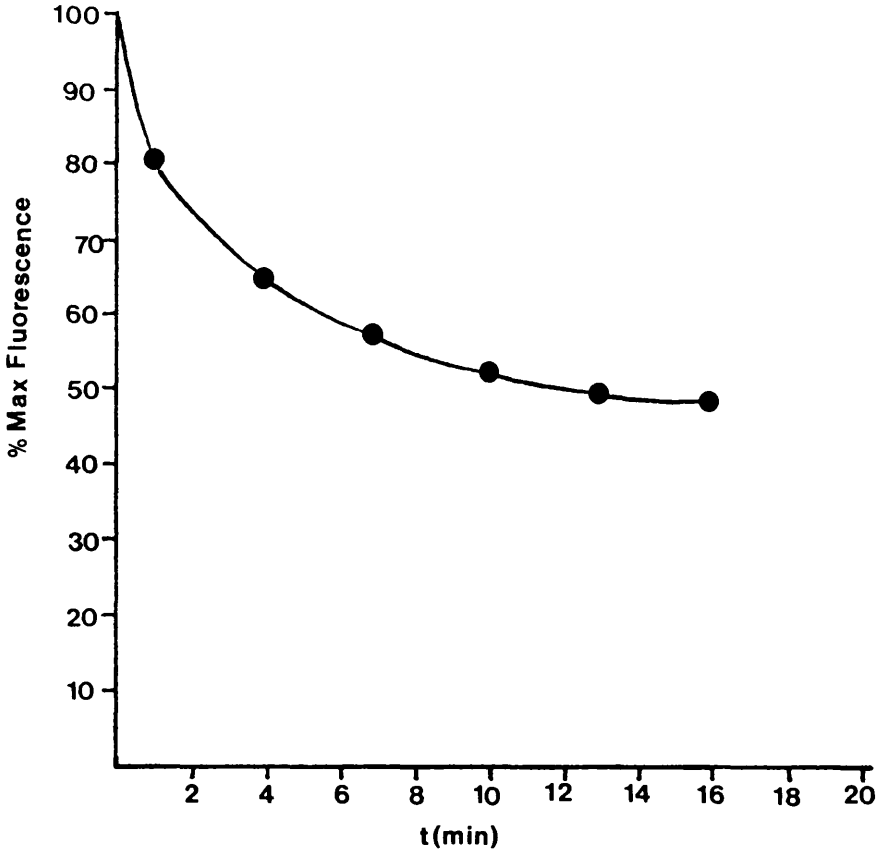


Figure 7.2 Fluorescence decay

The decay of flavin fluorescence with time

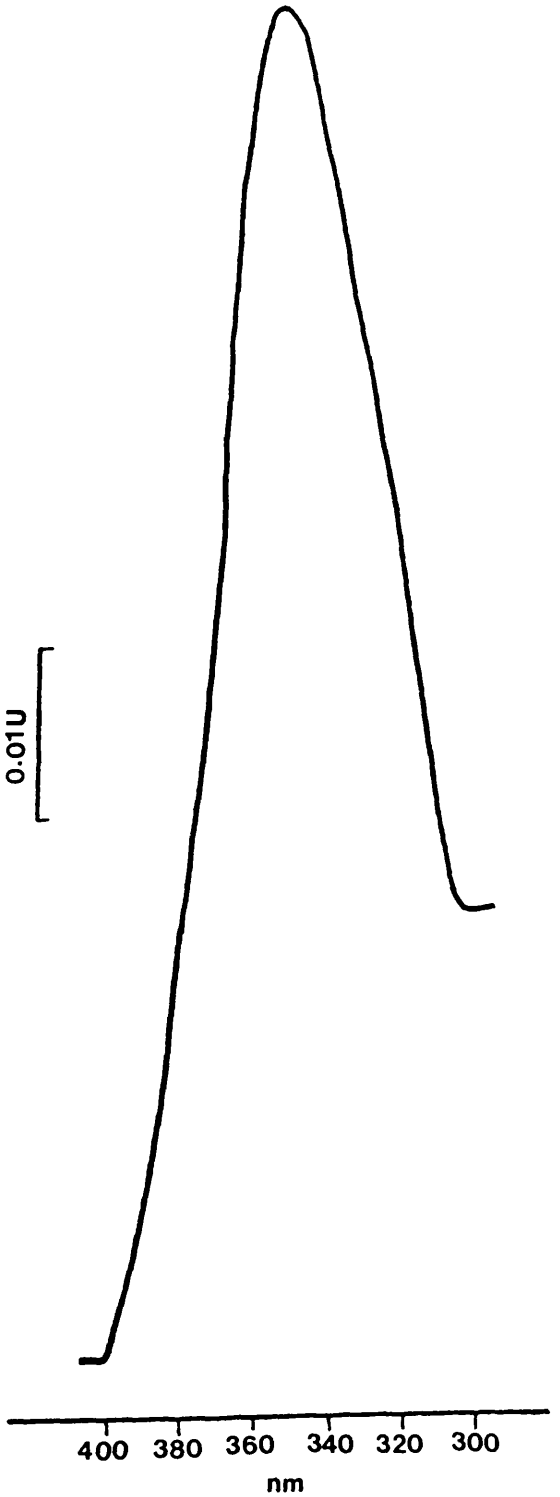


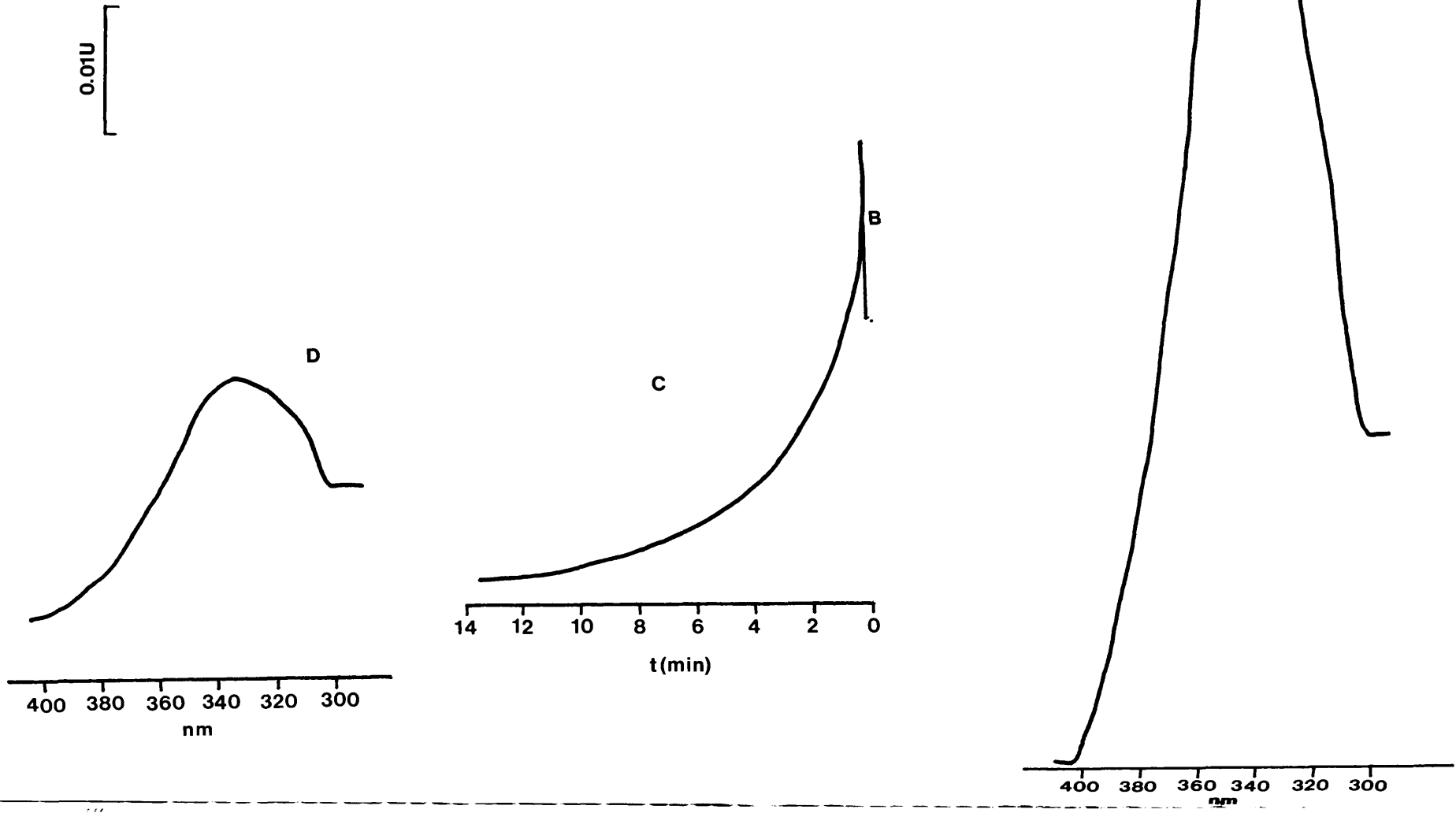
Figure 7.3 Aromatic fluorescence of the E. coli chorismate synthase

Solutions of E. coli chorismate synthase, 0.07mg/ml in 50mM-potassium phosphate buffer, pH7.0, were subjected to fluorescence spectroscopy as described in Section 2.10

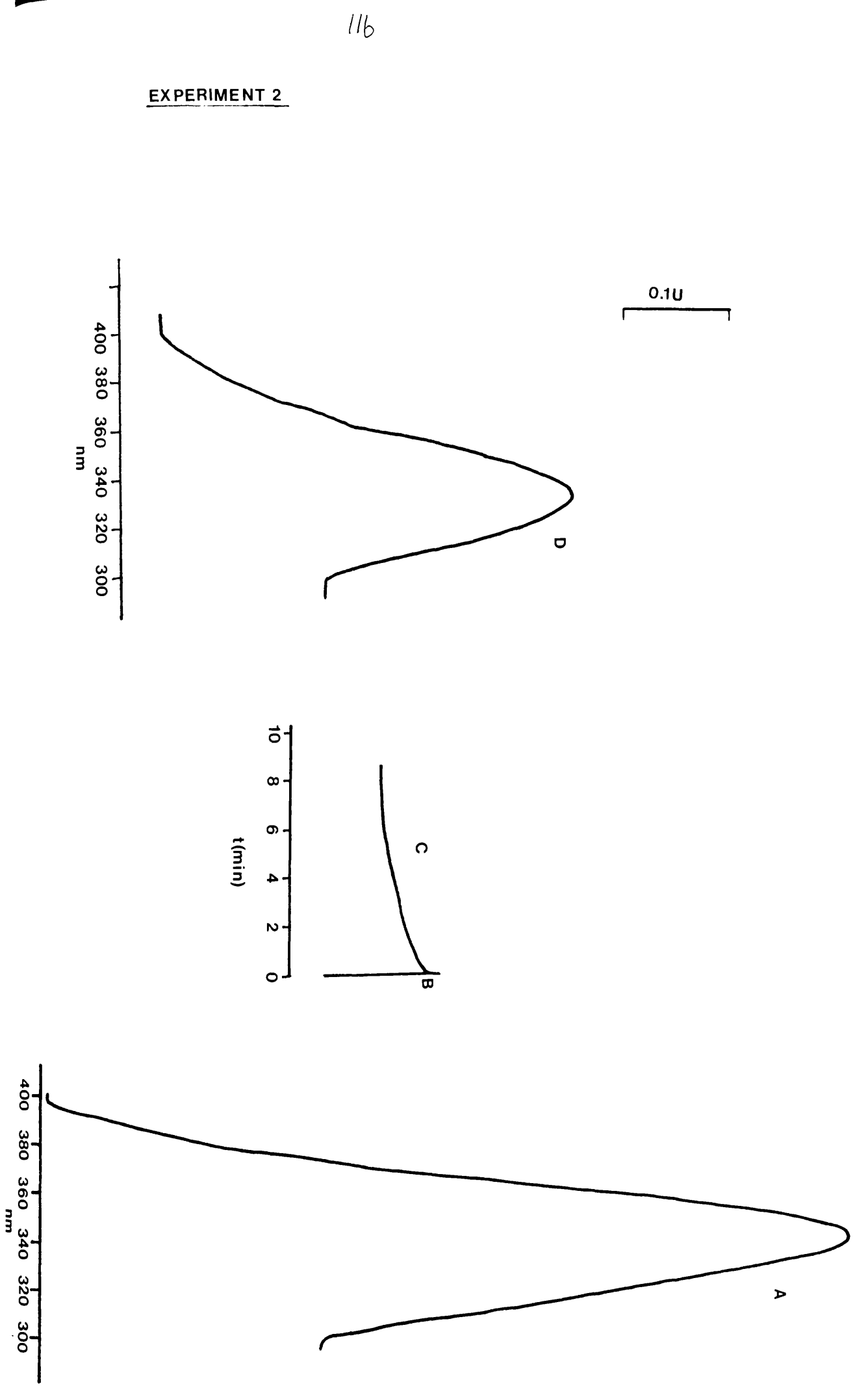
Figure 7.4 Quenching of aromatic fluorescence with EPSP and flavin

Solutions of E. coli chorismate synthase, 0.07 mg/ml in 50mM-potassium phosphate buffer, pH7.0, were subjected to fluorescence spectroscopy as described in Section 2.10. These two experiments monitor the effect of EPSP and flavin (FAD or FNM) on the aromatic fluorescence of E. coli chorismate synthase.
Experiment 1: A, emission spectrum; B, fluorescence at 340nm after the addition of FMN (final concentration 15uM); C, the decay of fluorescence at 340nm after the addition of EPSP (final concentration 40uM); D, emission spectrum at the end of the experiment.
Experiment 2: A, emission spectrum; B, fluorescence at 340nm after the addition of FAD (final concentration 15uM); C, the decay of fluorescence at 340nm after the addition of EPSP (final concentration 40uM); D, emission spectrum at the end of the experiment.

EXPERIMENT 1



EXPERIMENT 2



interaction. The lack of a 'slow phase' quenching with FAD may be attributed to steric hindrance caused by the adenine dinucleotide portion of the molecule. Since the *E. coli* chorismate synthase is active in the presence of either $FMNH_2$ or $FADH_2$ it would appear that these conformational changes may be necessary for the flavin to achieve a sterically favourable orientation to accept reducing equivalents from NADPH and/or to exclude further reaction with O_2.

7.5 The diaphorase activity

The *N.crassa* chorismate synthase exists as a bifunctional enzyme with both diaphorase and chorismate synthase activities residing on a single polypeptide chain. The diaphorase activity has been characterised in some detail (Welch *et al.*, 1974; Boocock, 1983). During the synthesis of chorismate, reducing equivalents are NOT consumed stoichiometrically, but in the absence of substrate (EPSP) a flavin dependent reductase activity can be assayed. Suitable electron acceptors e.g. DCPIP or cyt c can be reduced in the presence of FMN and NADPH. In the absence of such electron acceptors oxygen is not reduced. Both the cyt c and DCPIP reductase activities are 95% inhibited by 25uM-EPSP. The intact diaphorase/chorismate synthase complex found in *B. subtilis* also exhibits a flavin dependent oxidation of NADPH but the isolated diaphorase subunit shows a flavin independent DCPIP reductase activity. Boocock suggested this was consistent with a flavin binding site present within the chorismate synthase domain/subunit which received reducing equivalents from a spatially distinct NADPH binding site in the reductase domain/subunit. The monofunctional *E. coli* chorismate synthase isolated in this study was found to

contain bound flavin and a possible flavin binding site (see Chapter 5). This evidence is consistent with the proposed flavin binding chorismate synthase domain described above.

7.6 pH optima of the N.crassa chorismate synthase

pH effects can often give valuable information as to which ionisations are important in governing enzyme reaction mechanisms. Simplistically the N.crassa chorismate synthase can be regarded as possessing three enzyme activities which can be studied individually; 1. a diaphorase or flavin reductase, 2. a reaction that synthesises chorismic acid and 3. an overall reaction involving the sum of activities 1 and 2 above. Reactions 1 and 3 can be studied easily in continuous aerobic assays. The effect of pH on these two reactions was investigated. Preliminary plots of V_m against pH gave a pH optimum of 7.0 for the overall reaction and 8.5 for the diaphorase (cyt c reductase) activity (Figure 7.5). This disparity in pH optima means that, when chorismate synthesis is operating maximally, flavin reduction is only functioning at 40% efficiency. Such a situation would account for the remaining lag phase in chorismate synthesis even after preincubation of the enzyme with EPSP and FMN (Boocock, 1983). The important pK values for each reaction were determined from plots of $\log_{10} V_m$ against pH (Figure 7.6). The chorismate synthesis reaction showed two important ionisations (pK_1 =6.3 and pK_2=8.8) with a significant plateau of activity between these points. The diaphorase (cyt c reductase) activity also possessed two pK values but these were much closer in value (7.8 and 8.6).

Figure 7.5: pH optima of the N. crassa chorismate syntnase

Chorismate synthase and diaphorase activities were assayed at 0.5pH intervals in the following buffers: piperazine HCl (pH5.0-6.0); Bis-tris-HCl (pH6.0-7.0); Tris-HCl (pH7.5-8.5); Ethanolamine-HCl (pH 9.0-9.5). Buffer concentrations were 50mM and all buffers contained 50mM-KCl.

A, Chorismate synthase pH optimum: 8mU of highly purified N. crassa chorismate syntnase was incubated at each pH value for 4 minutes at 25°C. The incubation cocktail contained FMN (10uM) and NADPH (20uM). Assays were initiated by the addition of EPSP to a final concentration of 50uM and enzyme activities were calculated after the reaction had achieved a linear rate.

B, Diaphorase pH optimum: 8mU of highly purified N. crassa chorismate syntnase was incubated at each pH value for 4 minutes at 25°. The incubation cocktail contained FMN (10uM) and cytochrome c (0.5mg/ml). Assays were initiated by the addition of NADPH to a final concentration of 50uM and enzyme activities were calculated after the reaction had achieved a linear rate.

Each point is an average of two experimental values. Duplicates did not vary by more than 5%. Similar experiments were conducted with substrate concentrations twice those described above. This did not result in any significant increase in enzyme activity (Chorismate synthase, or diaphorase) at any of the pH intervals tested.

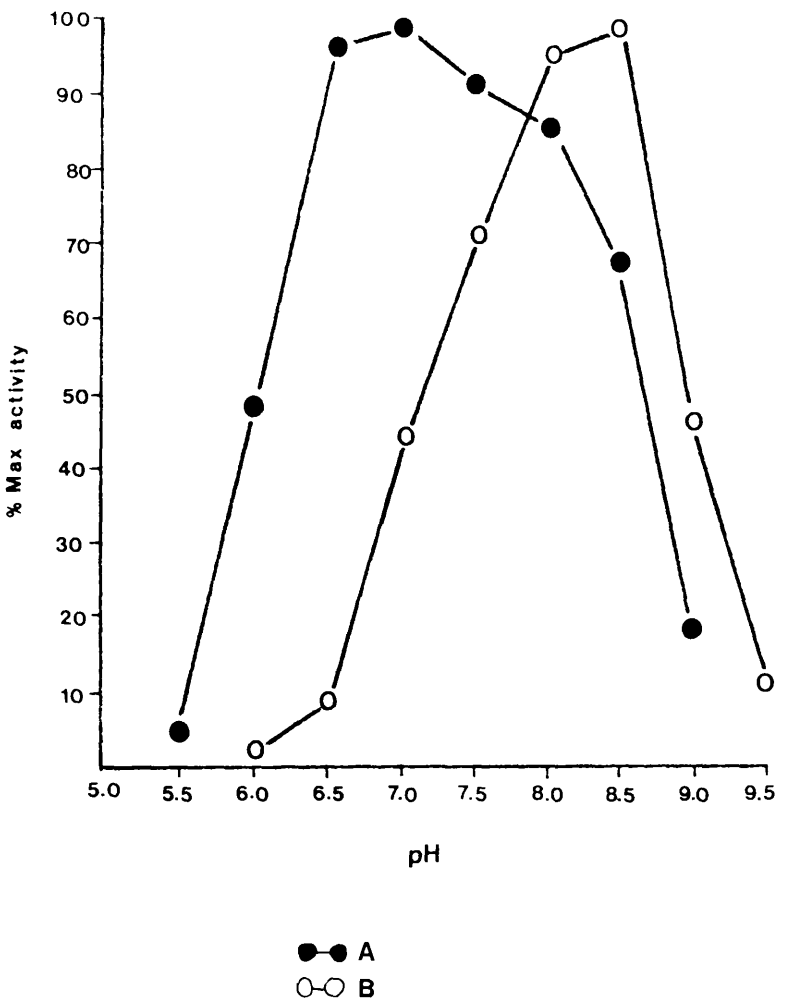
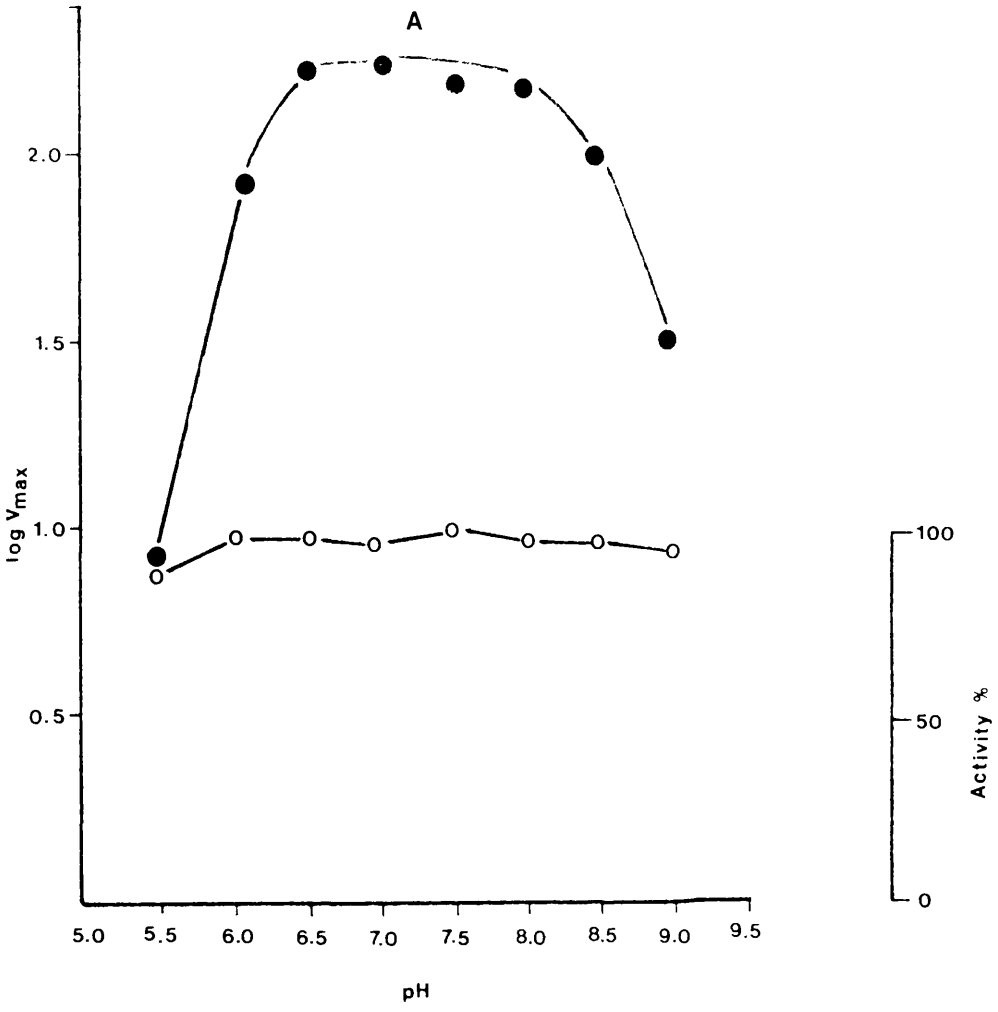
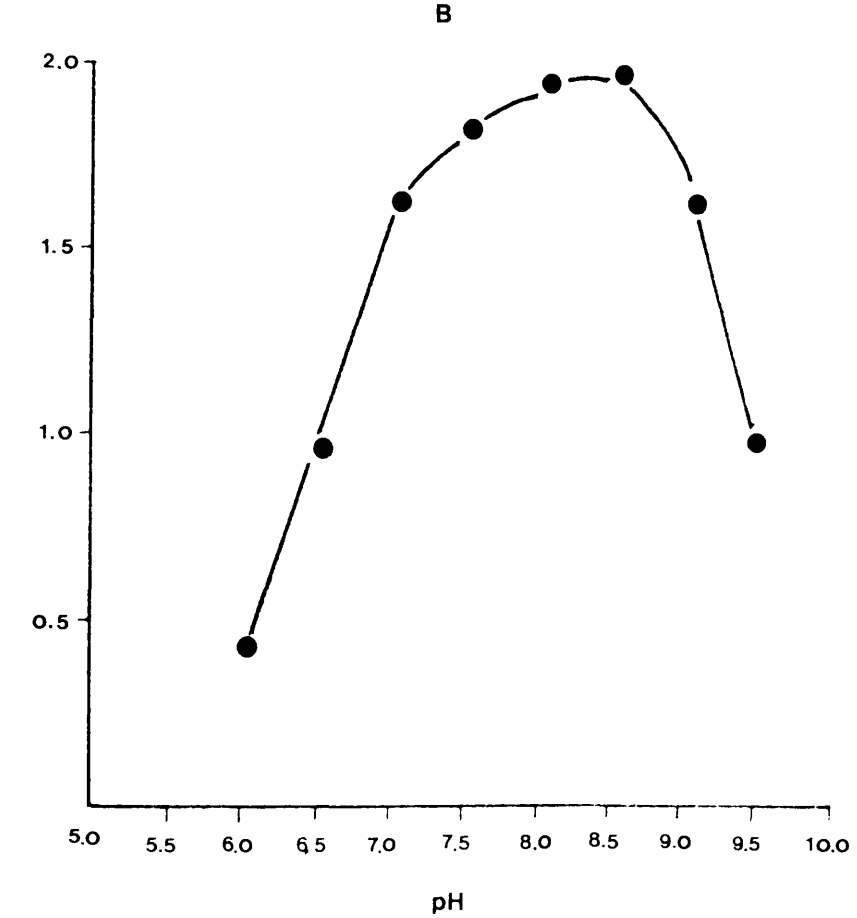

100
90
80
70
60
50
40
30
20
10
% Max activity
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH
A
B

Figure 7.6 A plot of $\log_{10} V_m$ against pH

A, chorismate synthase activity
B, diaphorase activity
Each point was derived from the pH optima experiment described in Figure 7.5.


A
2.0
1.5
1.0
0.5
log V_{max}
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH
100
50
0
Activity %


B
2.0
1.5
1.0
0.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

7.7 Possible catalytic roles for reduced flavin

The intimate role of the reduced flavin cofactor in the synthesis of chorismic acid is well supported by experimental evidence in this study and in previous work (Morell et al., 1967; Welch et al., 1974; Boocock, 1983). The catalytic role for such a cofactor is not clear. Preliminary data suggested that the reduced flavin was important in activating chorismate synthase by reducing an oxygen sensitive iron(II) centre or a disulphide bridge at the catalytic site of the enzyme (Morell et al., 1967; Welch et al., 1974). Experimental evidence for these hypotheses was not conclusive and it has been speculated that there is neither an iron(II) centre nor a reduced disulphide group at the active site, but that reduced FMN itself is directly involved in the reaction mechanism (Boocock, 1983).

7.7.1 Sensitivity to NEM

The purified N.crassa chorismate synthase from this study was found to be highly sensitive to thiol directed reagents. Less than 20% of original activity remained after incubation with 0.25mM-NEM. Substrates (EPSP and FMN) could partially protect the enzyme from inactivation (Figure 7.7). These losses in chorismate synthase activity were found to be consistent with equivalent losses of diaphorase (cyt c reductase) activity. A working assay of the N. crassa chorismate synthase was found to be 90% inhibited (after 2 min) when para-chloromercurobenzoate (PCMB) was added to a final concentration of 1mM.

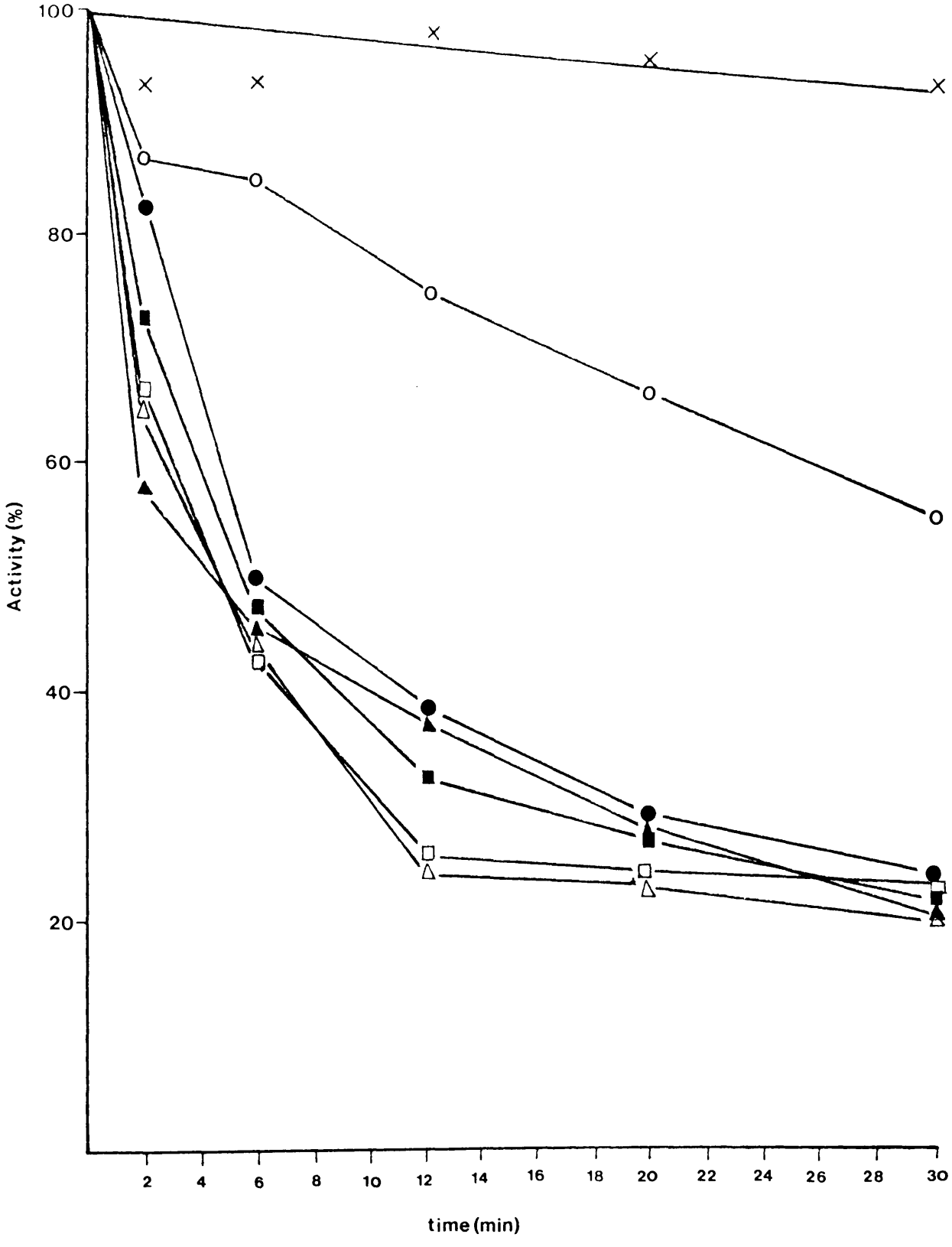
7.7.2 Sensitivity to metals and metal chelators

Neither the N. crassa nor the E.coli chorismate synthase from this study showed any significant sensitivity toward the

Figure 7.7 Sensitivity to NEM

N. crassa chorismate synthase was incubated with NEM. The final incubation cocktail was 200ul and contained (final concentrations), 64mU of highly purified enzyme, 250uM-NEM and the relevent protecting ligands in 50mM-TEA.HCl-KOH buffer pH7.0. The incubation was carried out at 25°C and at various time-points aliquots of the cocktail were removed and added to a complete chorismate synthase assay cocktail (Section 2.5.1). Chorismate synthase activity was monitored continuously at 275nm.

	Protecting ligand(s)	final concentration
O-O	EPSP+FMN	50uM, 10uM
□-□	EPSP+FAD	50uM, 10uM
Δ-Δ	EPSP	50uM
●-●	EPSP+NADPH	50uM, 10uM
■-■	NADPH+FMN	10uM, 10uM
▲-▲	no ligands	- -
x-x	control (no NEM)	- -


100
80
60
40
20
Activity (%)
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
time (min)

powerful metal chelating agents EDTA and diethyldithiocarbamic acid (20 mins at 10mM chelator in 50mM- TEA/Cl.KOH, pH7.0, 25^{o}C). The N. crassa enzyme could not be activated by incubation with a range of metal ions. Enzyme (8mU) was incubated at 25 C for 4min in a 1ml cuvette in the presence of the metal ion under study. The incubation cocktail contained (final concentrations) 50mM-TEA/Cl.KOH, pH7.0, 50mM-KCl and metal ion 100uM. The assay was initiated with substrates (final concentrations) 20uM-NADPH, 10uM-FMN, and 50uM-EPSP. The metal solutions studied were; copper chloride, manganese chloride, zinc sulphate; nickel chloride; magnesium sulphate; iron chloride, iron sulphate; cobalt chloride and calcium chloride. Copper chloride was found to inhibit significantly chorismate synthase activity.

7.8 Discussion

The N. crassa chorismate synthase is active in the presence of chemically generated $FMNH_2$ or $FADH_2$, but $FMNH_2$ is a significantly better substrate. Under aerobic conditions with the physiological reductant NADPH FAD is a very poor substrate and weak chorismate synthase activity is seen only after a lag period of many minutes. This result is consistent with the substrate specificity of the flavin reductase activity associated with this enzyme. Perhaps surprisingly chorismate synthase activity in the presence of chemically generated $FADH_2$ is nearly twice that of the aerobic assay in the presence of FMN. The significance of this result is not clear. An obvious suggestion is that the strictly anaerobic conditions prevent any reoxidation of reduced flavin. but since the consumption of reducing equivalents in the aerobic assay is

negligible then this seems unlikely. Alternatively a proportion of the N. crassa chorismate synthase enzyme molecules may have lost the ability to generate reduced flavin, perhaps by proteolytic damage of the diaphorase activity during purification. Incubation with chemically reduced flavin would allow these proteolysed enzymes to be 'reactivated'. Considering the great efforts made to prevent proteolysis and the presence of only one species of enzyme in SDS PAGE of the purified enzyme, this explanation also seems unlikely. Perhaps then oxygen itself is an inhibitor of enzyme activity or it may be possible that the enzyme is conformationally more active under strictly anaerobic conditions.

Studies of the characteristic lags in the N. crassa chorismate synthase activation by Boocock (1983) suggested that the enzyme underwent conformational changes in the presence of EPSP and FMN together, and that these conformational changes 'activated' or 'primed' the enzyme molecule. The fluorometric analyses described in this study suggest that the same type of hypothesis is applicable to the E. coli chorismate synthase. EPSP has a quenching effect on both the fluors present in the E. coli enzyme. The residual bound flavin could be significantly quenched over a period of several minutes. Thus it appears that EPSP can bind to chorismate synthase at or near the bound flavin in the enzyme. The aromatic fluorescence showed a more complicated quenching response. EPSP alone did not quench this fluor. FMN or FAD could give a rapid quenching of aromatic fluorescence but a further slow-phase quenching could then be accomplished on addition of EPSP to the FMN-enzyme complex; the FAD-enzyme complex could not be quenched furtner. These data suggest that FMN and EPSP act in concert

causing a conformational change in the enzyme. The quenching of the residual flavin on the binding of EPSP could be explained by a conformational change creating greater contact between flavin and the protein, perhaps allowing the isoalloxazine ring to be shielded by an aromatic residue or residues near the flavin binding region. Such a quenching of flavin fluorescence has been demonstrated in the flavodoxins (Mayhew & Ludwig, 1975) and in various model systems (Mackenzie et al., 1969). The aromatic fluorescence of E. coli chorismate synthase is also markedly quenched by flavins (FMN or FAD) and it is tempting to suppose that this could involve direct molecular overlap of the side chains of some of these residues with the isoalloxazine ring of FMN and FAD. Again the further quenching of this fluorescence in the E. coli enzyme when measured in the presence of FMN and EPSP suggest an increase in molecular 'contact' between the enzyme and these substrate molecules . Interestingly the quenching of aromatic fluorescence in two flavodoxins, Clostridium MP and A. vinelandii appears to be a consequence of such a molecular interaction and in the case of the A. vinelandii flavodoxin quenching is probably associated with a single tryptophan located in the vicinity of the FMN (Ryan & Tollin, 1973).

The pH optima of the two activities catalysed by the N. crassa chorismate synthase were studied. The optima differ significantly suggesting several side-chain ionisations are important for catalytic activity, not all of which are likely to be common to both reactions. This is certain evidence for the presence of two distinct catalytic centres on the one polypeptide chain. Although distinct, both active sites would necessarily have to be close enough to allow the reduced flavin to participate in the formation of chorismic acid.

The *N. crassa* chorismate synthase was found to be very sensitive to low concentrations of the thiol directed agent NEM. Partial protection could be achieved with EPSP+FMN. The working assay was also 90% inhibited by PCMB. It is probable that the *N. crassa* enzyme possesses one or more free thiol groups but these are not catalytically active. The *N. crassa* enzyme showed no sensitivity toward metal chelation with EDTA and was not activated by a range of metal ions. There is not yet any evidence to suggest that reduced flavin is required to generate a catalytically active Fe^{2+} ion.

7.9 Summary

Reduced FMN is the preferred cofactor for both the enzymes under study. Only the *N. crassa* enzyme has the ability to efficiently reduce oxidised FMN. The *E. coli* flavin and aromatic fluorescence can be quenched by the substrates FMN, FAD and EPSP, but a significantly greater response is seen with FMN and EPSP. *In vivo* the *E. coli* chorismate synthase subunit is the binding site for the flavin cofactor (FMN). The *N. crassa* chorismate synthase is highly sensitive to thiol directed agents, but there is no conclusive evidence that this enzyme possesses a catalytically active thiol. Preliminary metal chelation and activation studies did not reveal a catalytic role for a metal cofactor.

CHAPTER 8 FINAL DISCUSSION AND FUTURE PROSPECTS

8.1 Introduction

Information is now available with which to compare the structural and catalytic properties of chorismate syntnases from a number of prokaryotic and eukaryotic organisms. The enzyme has been purified to homogeneity from N. crassa, B. subtilis and E. coli and detected in chloroplast extracts from P. sativum. The enzymes can be grouped according to their ability to generate the reduced flavin cofactor. Only the N. crassa and B. subtilis enzymes show this property. The subunit molecular weights of the three purified enzymes appear quite heterogeneous, although the N. crassa and E. coli enzymes both have a tetrameric quaternary structure. Table 8.1 summarises some of the structural and catalytic properties of tne cnaracterised enzymes.

8.2 Has the N. crassa chorismate synthase arisen from the fusion of two genes encoding separate catalytic activites?

Tne N. crassa chorismate synthase is a homotetramer and is bifunctional, showing chorismate synthase activity in the presence of exogenously supplied reduced flavin and diaphorase activity in tne absence of EPSP. The purified E. coli chorismate synthase from this study could not reduce flavin. The B. subtilis enzyme possesses both activities but these are found on two separable polypeptide chains, the smaller of which has a subunit M_r of 13 000 and catalyses flavin reduction. The N. crassa and E. coli enzymes have some sequence homology, but the larger subunit size of the N. crassa chorismate synthase suggest that tne 'extra' 12kDa may

Table 8.1

Structural and catalytic properties of chorismate synthases

Source	Subunit M_r	Quaternary structure	Diaphorase activity	Preferred flavin
N.crassa[1]	50 000	tetramer	Yes	FMN
B.subtilis[2]	24 000[a] 17 000[b] 13 000[c]	-	Yes (a+c)	FMN/FAD
E.coli[3]	38 000	tetramer	None detectable	FMN
P.sativum[4]	no data	no data	None detectable	no data

Reference
1. This study.
2. Hasan & Nester (1978a, b, and c).
3. This study.
4. Mousdale & Coggins (1987).

be associated with the diaphorase activity. This hypothesis is numerically attractive as the B. subtilis flavin reductase subunit is of a similar size. It is conceivable then, that an E. coli-like chorismate synthase domain of 38kDA could have become fused to a smaller (12-13 kDa) flavin reductase to yield a new bifunctional polypeptide of 50kDa. The only flaw in this reasoning is the fact that the B. subtilis chorismate synthase domain has a much smaller subunit M_r of 24kDa. One could argue that the 38kDa E. coli enzyme is composed of a 24kDa chorismate syntnase domain and a catalytically inactive 12-13kDa diaphorase activity. However this alternative hypothesis appears less likely when one considers two important observations. Firstly, some of the shikimate pathway activites in B. subtilis are dependent on protein-protein interactions. Dehydroquinate synthase shows no activity unless complexed with both the chorismate synthase and flavin reductase subunits, and shikimate kinase is also inactive unless associated with the enzyme DAHP synthase. Both these enzymes have subunit molecular weights that are about 50% smaller than the corresponding activities in E. coli, and it may be necessary for protein-protein interactions to compensate for this deficiency in subunit size. Secondly, although the specific activity of the B. subtilis chorismate synthase in crude extracts is comparable to other chorismate synthases studied, the specific activity of the purified enzyme is nearly 35 times less than the highly purified N. crassa enzyme from this study (Table 3.3). This purified enzyme may have suffered proteolytic damage during the purification or perhaps lost some important protein-protein interactions.

Genetic studies have identified only a single complementation group of arom-3 (chorismate synthase) mutations

which exhibit extensive allelic complementation, as would be expected for a single N. crassa bifunctional chorismate synthase gene product (Rines et al., 1969).

8.3 The need for a bifunctional enzyme

The enzyme source of physiological reducing power for the monofunctional (E. coli and P. sativum) enzymes has not yet been identified. Some microorganisms, for example Aerobacter aerogenes and E. coli, have been shown to possess strong diaphorase activities (Bernofsky & Mills, 1966; Morell et al., 1967), and it is conceivable that in vivo the monofunctional chorismate synthases are able to obtain reduced flavin from such a source. Interestingly, cell-free extracts of N. crassa show very little diaphorase activity (D.M. Mousdale, unpublished work), and this may explain why the N. crassa chorismate synthase is found as a bifunctional enzyme in covalent association with a specific diaphorase activity.

8.4 What role for reduced flavin?

Reduced flavin is essential for chorismate synthase activity. Beyond this one can only speculate as to its catalytic function. Preliminary experiments reported in this thesis did not reveal any immediately obvious role for either a reducible metal cofactor or an active site thiol. The probability that flavin is an essential structure promoting component must be considered, but the enzyme's weak affinity for flavin in the absence of substrate, and the intimate relationship between EPSP and FMN binding, suggests a more direct catalytic role. The reduced flavin itself would offer a strongly nucleophilic environment within the enzyme and this

alone could act as the enzyme bound nucleophile in the two-step mechanism proposed by Onderka & Floss (Figure 1.5). A possible reaction mechanism involving reduced flavin is shown in Figure 8.1. If reduced flavin does have a direct catalytic role then substituting flavin with artificial analogues may be useful in determining which sites in the flavin cofactor are important for catalytic activity. If the reaction does proceed via an N-5 adduct, then replacing FMN with 5-deazaflavin (N-5 replaced with carbon) would be expected to abolish catalytic actvity. This analogue has been shown to be a useful tool in understanding some flavoprotein reaction mechanisms (Hemmerich et al., 1977; Ghisla & Massey, 1986).

8.5 Future work

The availability of reasonable quantities of both the E. coli and N. crassa chorismate synthases opens the way for further structural and kinetic analyses of there two fascinating enzymes. Partial proteolysis of the N. crassa enzyme with amino and/or carboxypeptidases may allow the diaphorase domain to be located within the bifunctional polypeptide. Ultimately the cloning and sequencing of the N. crassa chorismate synthase gene will allow the primary structure of both enzymes to be compared. The use of artificial flavins as active site probes, and sophisticated spectroscopic techniques will allow the unusual reaction mechanism to be studied in greater detail.

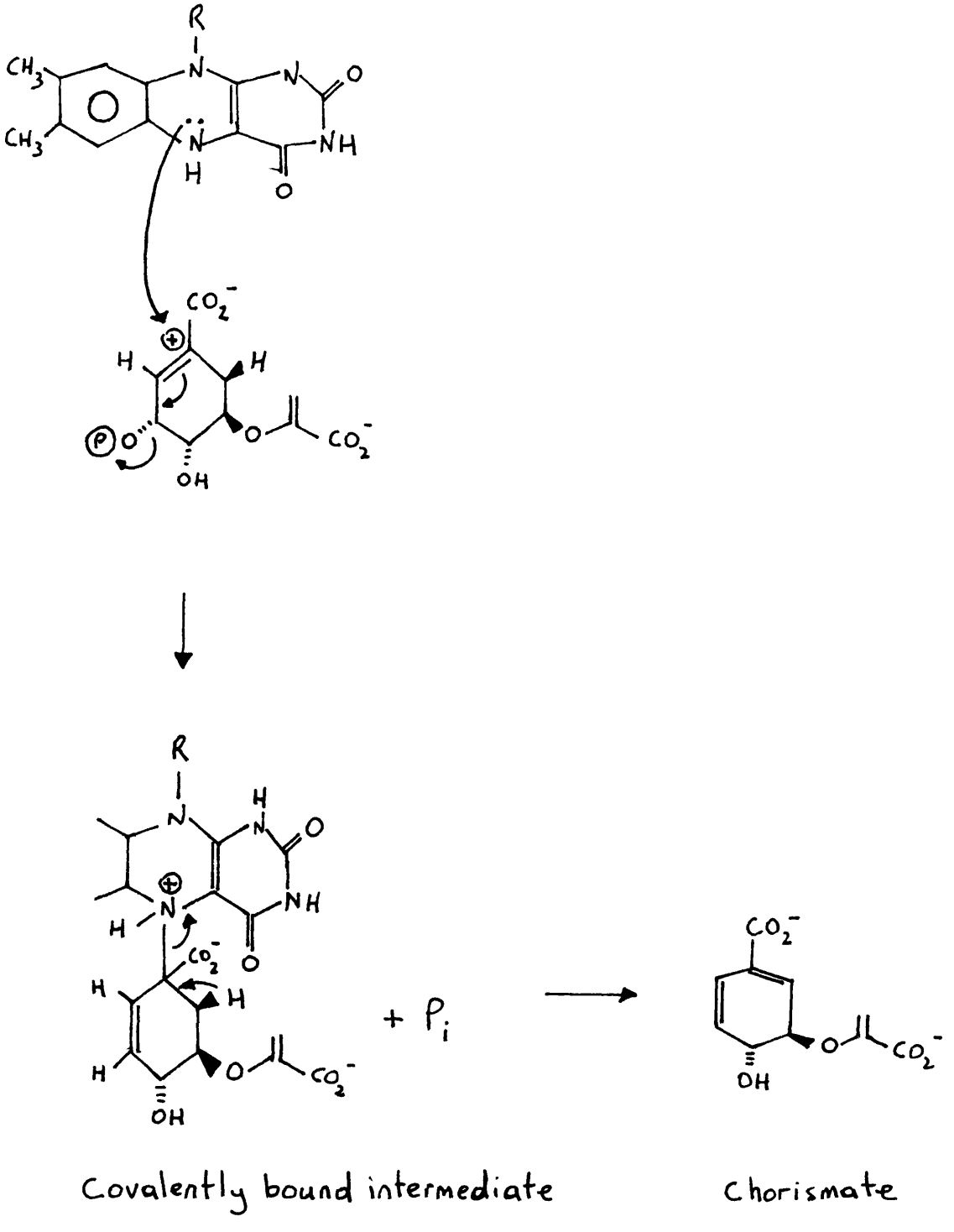


Figure 8.1 A possible reaction mechanism for chorismate synthase

Nucleophilic attack by the reduced flavin forms a flavin-EPSP intermediate with the loss of phosphate. A rearrangement then follows, with the return of electrons to the flavin molecule releasing chorismate and a proton.

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