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Isolation of a bifunctional domain from the pentafunctional *arom* enzyme complex of *Neurospora crassa*

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Limited proteolysis of the *arom* enzyme complex of *Neurospora crassa* by trypsin or subtilisin yielded a stable fragment of M_r 68000. This fragment, which was purified by two-dimensional polyacrylamide-gel electrophoresis, was shown by activity staining to contain the shikimate dehydrogenase active site, and by substrate labelling with 3-dehydroquinate and NaB^3H_4 to contain the 3-dehydroquinase active site. The fragment thus constitutes a bifunctional domain containing the two enzymic activities that are known, from genetic evidence, to be located adjacently at the *C*-terminal end of the pentafunctional *arom* polypeptide.

The *arom* enzyme complex of the mould *Neurospora crassa* catalyses five reactions on the early common pathway of aromatic-amino-acid biosynthesis (see Scheme 1). When purified rapidly in the presence of proteinase inhibitors the *arom* complex was found to be composed of two subunits, each of M_r 165000 (Lumsden & Coggins, 1977; Gaertner & Cole, 1977). Peptide mapping demonstrated that these subunits are identical (Lumsden & Coggins, 1978), establishing the pentafunctional character of the *arom* polypeptide chain.

Limited proteolysis has proved a useful tool in the study of the structure of multifunctional enzymes as it offers a method of isolating functional domains (Kirschner & Bisswanger, 1976; Dileepan *et al.*, 1978; Tan & McKenzie, 1979; Dautry-Varsat & Garel, 1978; McCarthy & Hardie, 1983). The observation that extensive proteolysis of the purified *arom* enzyme complex did not lead to loss of the shikimate dehydrogenase activity (Lumsden & Coggins, 1977) suggested that after limited proteolysis it should be possible to isolate a fragment of the complex containing at least this activity. In the present paper we describe how treatment of the *arom* enzyme complex with bovine trypsin and other proteinases leads to the formation of a bifunctional fragment. This fragment is shown to contain both the 3-dehydroquinase and shikimate dehydrogenase activities, and genetic evidence (Giles *et al.*, 1967) indicates that it is derived from the *C*-terminal end of the pentafunctional *arom* polypeptide.

Abbreviations used: Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; Bz-Arg-Nan, α-*N*-benzoyl-DL-arginine 4-nitroanilide.

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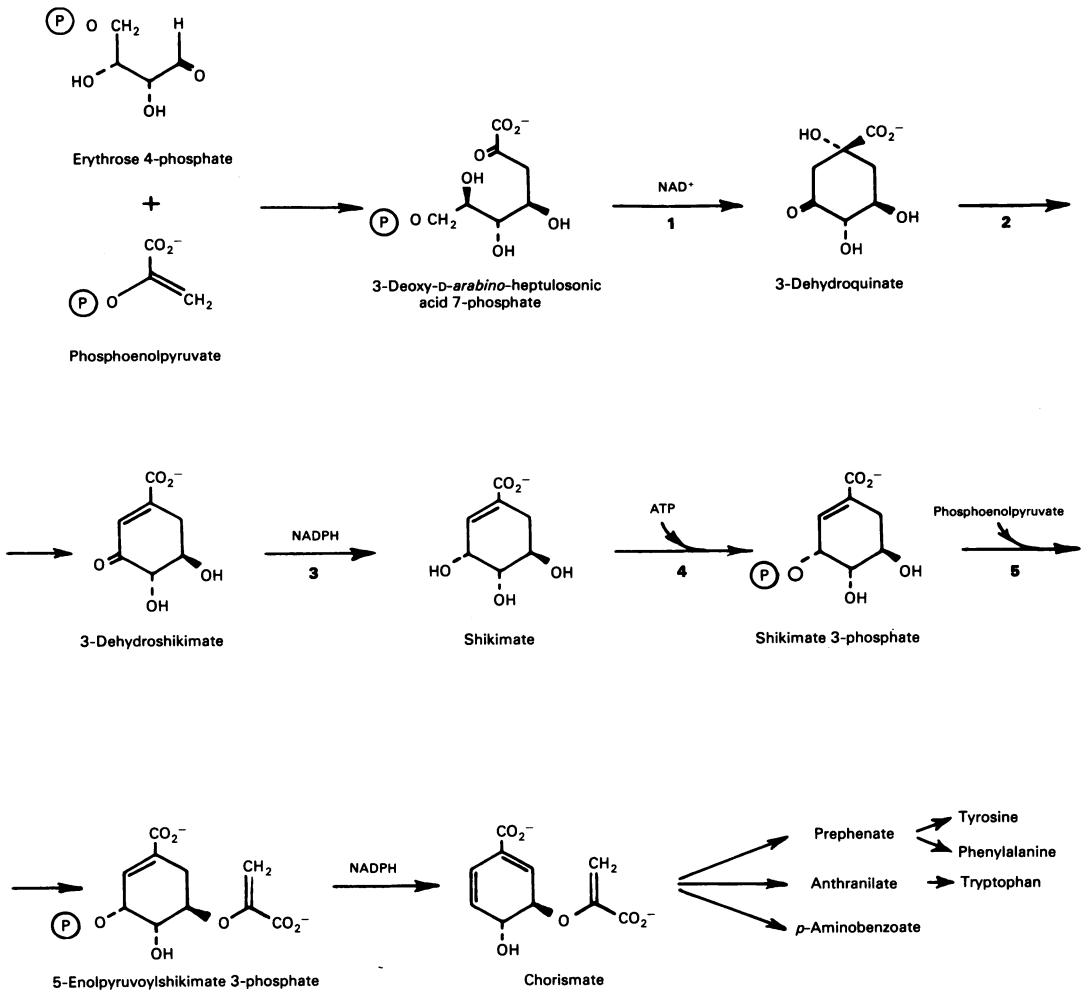
Materials and methods

Chemicals

Blue Dextran, Bz-Arg-Nan, subtilisin (subtilopeptidase A), elastase, soya-bean trypsin inhibitor, lima-bean trypsin inhibitor and thioglycollic acid were obtained from Sigma (London) Chemical Co., Poole, Dorset, BH17 7NH, U.K.; phosphoenolpyruvate, rabbit muscle aldolase and rabbit muscle pyruvate kinase/lactate dehydrogenase and all nucleotides were from Boehringer Corporation (London) Ltd., Lewes, East Sussex BN7 1LG, U.K.; bovine trypsin (Tos-Phe-CH_2Cl-treated) and papain were from Worthington Biochemical Corporation, Freehold, NJ 99727, U.S.A.; agarose–soya-bean trypsin inhibitor was from Miles Laboratories Ltd., Slough, Berks. SL2 4LY, U.K. and Sepharose 4B was from Pharmacia (G.B.) Ltd., Hounslow, Middx. TW3 1NE, U.K.

Blue Dextran–Sepharose was prepared as described by Ryan & Vestling (1974). Urea used for electrophoresis was freshly recrystallized from boiling ethanol. Dimethyl suberimidate dihydrochloride and cross-linked aldolase were prepared as described previously (Lumsden & Coggins, 1977). All other materials were purchased from the sources listed in the appropriate references or from BDH Chemicals, Poole, Dorset, U.K.



Scheme 1. *Reactions of the early common pathway of aromatic-amino-acid biosynthesis*

The *arom* enzyme complex, which catalyses the five numbered reactions, is responsible for the conversion of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate into 5-enolpyruvoylshikimate 3-phosphate. The enzymes involved are: **1**, 3-dehydroquinate synthase (EC 4.6.1.3); **2**, 3-dehydroquinase (EC 4.2.1.10); **3**, shikimate dehydrogenase (EC 1.1.1.25); **4**, shikimate kinase (EC 2.7.1.71); **5**, 5-enolpyruvoylshikimate-3-phosphate synthase (EC 2.5.1.19).

Purification of the arom enzyme complex

Arom enzyme complex was isolated from *Neurospora crassa* strain 74-OR23-1A as described by Lumsden & Coggins (1977), except that preparations were routinely carried out on 100g (dry weight) of freeze-dried mycelia, and the deoxyribonuclease step was replaced by a simple incubation at 37°C for 90min. The final chromatographic column was changed from cellulose phosphate to Blue Dextran–Sepharose as follows. The fractions from the DEAE-cellulose chromatography step which contained shikimate dehydrogenase activity were pooled (approx. 80ml) and applied overnight to a column of Blue Dextran–Sepharose 4B (4ml bed volume) previously equilibrated with 0.1M-Tris/HCl buffer, pH7.5, containing 0.4mM-dithiothreitol and 1.2mM-phenylmethanesulphonyl fluoride (buffer A). The column was washed with buffer A containing 0.5 M-KCl until the A_{280} of the eluate was zero. The *arom* complex was then eluted with buffer A containing 1.5M-KCl. The column was operated at 10ml/h; 4ml fractions were collected until the 1.5M-KCl step, when 2ml fractions were collected. The fractions containing shikimate dehydrogenase activity were pooled and dialysed against 50mM-sodium phosphate (pH7.5)/0.4mM-dithiothreitol/1.0mM-benzamidine/50% (v/v) glycerol.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed as described previously (Lumsden & Coggins, 1977). Radioactivity was detected in gel slices as described by Bates *et al.* (1975). Electrophoresis in the absence of sodium dodecyl sulphate was also performed as described previously (Lumsden & Coggins, 1977) except that the gels contained 1 M-urea. The samples were not treated with urea before electrophoresis. After electrophoresis each gel was washed for 30 min at 20°C with 10 ml of 25 mM-Tris/HCl buffer, pH 9.0 (buffer B). Shikimate dehydrogenase activity was then detected as described by Lumsden & Coggins (1977). In some experiments electrophoresis was also performed in the presence of 8 M-urea. In this case 0.02% (w/v) thiogylcollic acid was added to the anodic (upper) well buffer and the samples were also made 8 M in urea by addition of solid urea. Urea-treated samples were incubated for 20 min at 25°C before electrophoresis, which was carried out for 135 min at 3 mA/gel. Before staining for shikimate dehydrogenase activity each gel was incubated first with 100 ml of buffer B containing 0.5 mM-dithiothreitol for 50 min at 20°C and then with 10 ml of buffer B for 30 min at 20°C.

Before second-dimension polyacrylamide-slab-gel electrophoresis in the presence of sodium dodecyl sulphate, the tube gels which had been run either in the presence of 1 M-urea or 8 M-urea were incubated for a total of 60 min. with three changes of 10 ml each of 0.1 M-sodium phosphate (pH 6.5)/0.1% (w/v) sodium dodecyl sulphate/0.5% (w/v) 2-mercaptoethanol. The second-dimension polyacrylamide-slab-gel electrophoresis in the presence of sodium dodecyl sulphate was performed as described by Coggins *et al.* (1977).

The M_r values of the proteolytic fragments were estimated from plots of $\log M_r$ against R_F. The standard proteins used to construct the plots were sperm-whale myoglobin (M_r 17200), rabbit muscle aldolase (M_r 40000), *Escherichia coli* pyruvate dehydrogenase multienzyme complex E1 (M_r 100000), E2 (M_r 82000) and E3 (M_r 56000) (Danson *et al.*, 1979) and intact *arom* polypeptide (M_r 165000) (Lumsden & Coggins, 1977).

Densitometer traces of Coomassie Blue-stained gels were obtained at 600 nm by using a Gilford model 2520 gel scanner. The areas under the peaks of the densitometer traces were computed by using a Summagraphics 1D-CTR Data Tablet interfaced with a Digital Equipment PDP 11/34 computer.

Enzyme assays

Shikimate dehydrogenase (shikimate:$NADP^+$ 3-oxidoreductase, EC 1.1.1.25) and 3-dehydroquinase (3-dehydroquinate hydrolyase, EC 4.2.1.10) were assayed as described previously (Lumsden & Coggins, 1977).

Shikimate kinase (ATP:shikimate 5-phosphotransferase, EC 2.7.1.71) was assayed by coupling to pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27). The assays were carried out at 37°C in a total volume of 1 ml and the oxidation of NADH at 340 nm ($\varepsilon = 6180$ litre $\cdot$ mol^{-1} $\cdot$ cm^{-1}) was monitored with a Unicam SP. 8000 spectrophotometer with a slave recorder attachment. The reaction mixture contained 1 mM-shikimic acid, 1 mM-phosphoenolpyruvate, 0.2 mM-NADH, 2.5 mM-ATP, 0.1 M-KCl, 20 mM-$MgCl_2$, 50 mM-triethanolamine hydrochloride, pH 7.2, 2 units of pyruvate kinase and 0.7 unit of lactate dehydrogenase. Trypsin was assayed by the method of Erlanger *et al.* (1961). One unit of enzyme activity is defined as the amount of enzyme that catalyses the conversion of 1 μmol of substrate/min.

Labelling with NaB^3H_4

3-Dehydroquinase active sites were labelled with ^3H by reduction of the Schiff's-base intermediate obtained when enzyme and substrate interact with NaB^3H_4 (Butler *et al.*, 1974). To form the covalent intermediate, 0.1 mM-3-dehydroquinate was added to the enzyme solution in 50 mM-sodium phosphate buffer, pH 7.5. The intermediate was then reduced, at 0°C, by adding portions of NaB^3H_4 (sp. radioactivity 555 mCi/mmol) until the 3-dehydroquinase activity had fallen to approx. 10% of the initial value. Excess labelling reagents were then removed by gel filtration.

Limited proteolysis

Limited proteolysis of the *arom* enzyme complex with trypsin was performed at 25°C in 50 mM-sodium phosphate, pH 7.5, containing 0.4 mM-dithiothreitol and 1.0 mM-EDTA. The final trypsin concentration was 10 μg/ml (7 m-units/ml) and the final concentration of the *arom* enzyme complex was 250 μg/ml. Some proteolysis experiments were also carried out in the presence of specific concentrations of shikimate and $NADP^+$ or other ligands. Proteolysis with trypsin was terminated either (1) by the addition of soya-bean trypsin inhibitor in a 3-fold excess by weight over trypsin or (2) by passing the solution through a 1 ml column of immobilized soya-bean trypsin inhibitor or (3) in analytical experiments, where only enzyme activities were monitored, by dilution into an assay cuvette.

Limited proteolysis with subtilisin was performed at 0°C in 50 mM-sodium phosphate, pH 7.5, containing 0.4 mM-dithiothreitol and 1.0 mM-EDTA. The final subtilisin concentration was 3 μg/ml and proteolysis was terminated by the addition of phenylmethanesulphonyl fluoride [added as a 6 mg/ml

solution in 95% (v/v) ethanol] to a final concentration of 1.2mM.

Limited-proteolysis experiments with chymotrypsin, papain and elastase were carried out at 25°C by using the buffer conditions described for subtilisin. The final proteinase concentrations were chymotrypsin, 1.25μg/ml, papain, 5μg/ml, and elastase, 5μg/ml. Proteolysis was stopped in the case of chymotrypsin by the addition of lima-bean trypsin/chymotrypsin inhibitor to a final concentration of 12.5μg/ml and for papain and elastase proteolysis was stopped by boiling for 2min.

Cross-linking with dimethyl suberimidate

This was carried out as described previously (Lumsden & Coggins, 1977).

Estimation of protein concentration

The concentration of purified *arom* enzyme complex was estimated spectrophotometrically using a specific absorption coefficient ($A_{280}^{1\%}$) of 11.0 (Lumsden & Coggins, 1978).

Results

Purification of the arom enzyme complex from Neurospora crassa

The purification procedure of Lumsden & Coggins (1977) was modified as described in the Materials and methods section. The elution profile of the Blue Dextran–Sepharose column is given in Fig. 1. The purified enzyme was homogeneous as judged by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (see Fig. 2) and by polyacrylamide-gel electrophoresis under native conditions (results not shown). In six purifications carried out on the same scale, yields varied from 26 to 32% and specific activities from 61 to 91 units/mg of protein. These yields are better than those obtained in our previous purification procedure (Lumsden & Coggins, 1977) and the specific activities are comparable.

Limited proteolysis

The principal objective of this work was to isolate and characterize a proteolytic fragment from the *arom* polypeptide which carried at least the shikimate dehydrogenase activity. *Arom* enzyme complex (250μg/ml) was incubated with trypsin (10μg/ml) and, after 20min, proteolysis was terminated by addition of soya-bean trypsin inhibitor. This treatment led to 85% loss of shikimate kinase activity, but the shikimate dehydrogenase and 3-dehydroquinase activities remained at 96% and 100% of their initial levels respectively. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that there was scarcely any intact *arom* polypeptide remaining and the major product of proteolysis was

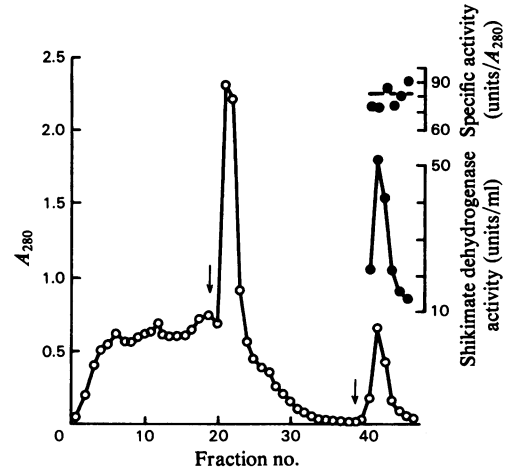


Fig. 1. *Chromatography of the arom enzyme complex on Blue Dextran–Sepharose 4B (final stage of purification)*
Enzyme from the second DEAE-cellulose column (280 units of shikimate dehydrogenase activity in 78ml) was applied directly to the column (bed volume 4ml) which had been equilibrated with buffer A. At the first arrow, buffer A containing 0.5M-KCl was applied. At the second arrow, buffer A containing 1.5M-KCl was applied. The flow rate was 10ml/h and either 24-min (1–19) or 12-min (20–47) fractions were collected. The lines show A_{280} (○) and shikimate dehydrogenase activity (●).

a polypeptide of M_r 68000 which presumably carried both the 3-dehydroquinase and shikimate dehydrogenase activities.

To find out whether the shikimate kinase could also be stabilized against proteolytic inactivation some trypsin treatments were carried out in the presence of various ligands which might be expected to afford some protection to the shikimate kinase active site. Proteolysis in the presence of the substrates of the shikimate kinase reaction and in the presence of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP), which had been reported by Vitto & Gaertner (1978) to stabilize the enzyme activities of the *arom* polypeptide against inactivation by proteolysis, gave the results presented in Table 1. In every case the rate of loss of shikimate kinase activity showed pseudo-first-order kinetics. Shikimate alone gave some protection against inactivation, but MgATP was much less effective. A mixture of shikimate and MgATP was more effective than shikimate alone, whereas shikimate plus MgADP, which might be expected to form a dead-end complex with the enzyme, was even more effective. DAHP afforded only slight protection of the shikimate kinase activity. $NADP^+$ gave virtually no protection, but the combination shikimate plus

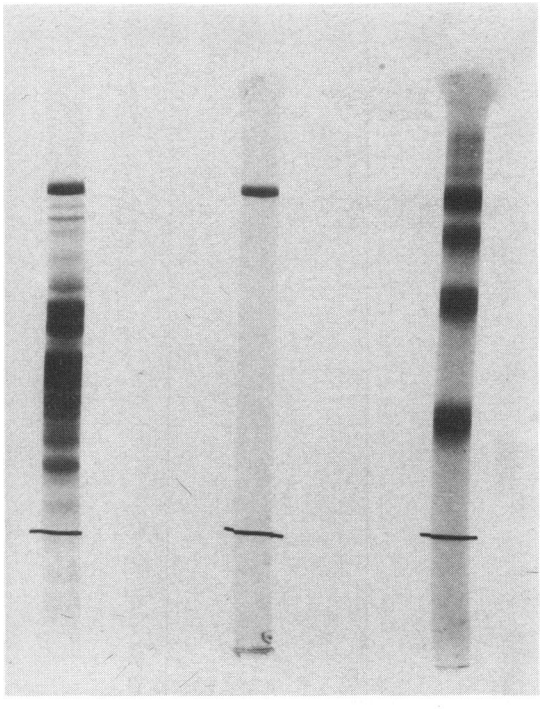


Fig. 2. *Polyacrylamide-gel electrophoresis of arom enzyme complex purified by chromatography on Blue Dextran–Sepharose 4B*

The 5% gels shown were run in the presence of sodium dodecyl sulphate as described in the Materials and methods section. Track (*a*) shows a sample (100 μl) of the pool from the second DEAE-cellulose column which was applied to the Blue Dextran–Sepharose 6B column. Track (*b*) shows 5 μg of *arom* enzyme complex purified on the Blue Dextran–Sepharose column. Track (*c*) shows rabbit muscle aldolase cross-linked with dimethyl suberimidate; the four prominent bands have M_r values of 40000, 80000, 120000 and 160000.

Table 1. *Effect of protecting ligands on the inactivation of shikimate kinase activity of the arom enzyme complex by trypsin*

Purified *arom* enzyme complex was incubated with trypsin as described in the Materials and methods section and samples were withdrawn at intervals and assayed for enzyme activity. In parallel control experiments in which no trypsin was added there was no detectable loss of shikimate kinase activity during the 60 min duration of the experiment. In all cases pseudo-first-order inactivation occurred and the half-lives were measured from the linear semi-logarithmic plots.

Protecting ligand	Time for 50% loss of shikimate kinase activity (min)
None	3.6
1 mM-DAHP	6.2
10 mM-$MgCl_2$ + 1 mM-ATP	5.5
10 mM-$MgCl_2$ + 1 mM-ATP + 1 mM-shikimate	16.0
10 mM-$MgCl_2$ + 1 mM-ADP + 1 mM-shikimate	43.5
1 mM-Shikimate	11.0
5 mM-$NADP^+$	4.4
5 mM-Shikimate + 5 mM-NADP	26.0

$NADP^+$ (the substrates for the reverse shikimate dehydrogenase reaction) was better at protecting the shikimate kinase activity from proteolytic inactivation than any of the other ligands or ligand mixtures tried except shikimate plus MgADP. Although the half-life of the shikimate kinase activity could be increased more than 10-fold by using suitable protecting ligands, no means of stabilizing the activity completely against inactivation by trypsin was found.

In order to produce sufficient material for separation and analysis of the fragment carrying shikimate dehydrogenase activity, a large-scale proteolysis experiment was carried out in the presence of $NADP^+$ and shikimate (Fig. 3). The extent of proteolysis and the amount of shikimate kinase inactivation were less than in the preliminary experiment because of the presence of $NADP^+$ and shikimate during proteolysis. The resulting *arom* complex had retained essentially all its shikimate dehydrogenase and 3-dehydroquinase activity but had lost 52% of its shikimate kinase activity. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that a substantial amount of intact *arom* polypeptide (M_r 165000) remained and, in addition to the fragment of M_r 68000 observed in the preliminary experiment, there was a second major fragment of M_r 110000 (Fig. 3, gel *b*).

It was now necessary to establish whether the shikimate dehydrogenase and 3-dehydroquinase activities were associated with one or both of these major proteolytic fragments. Firstly 3-dehydroquinase active sites were labelled by treatment with 3-dehydroquinate and NaB^3H_4. Samples were subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 4) and ^3H was detected by cutting a gel into slices and counting the radioactivity. The results, shown in Fig. 4, clearly demonstrate the presence of a 3-dehydroquinase active site in the uncleaved *arom* polypeptide and in the fragment of M_r 68000, but not in the fragment of M_r 110000. The specific activities of the two labelled bands were estimated by measuring the peak areas on the densitometer trace for the gel

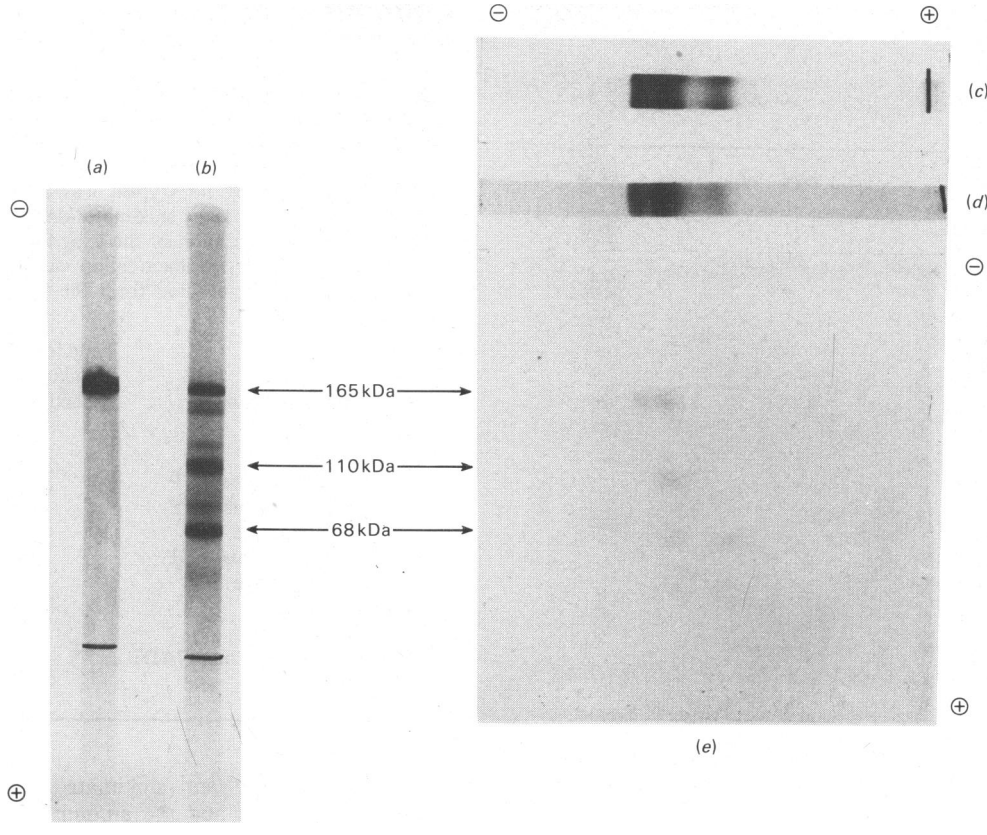


Fig. 3. *Polyacrylamide-gel electrophoresis of trypsin-treated arom enzyme complex*

Arom enzyme complex (1.75 mg) was incubated for 15 min at 25°C with trypsin as described in the Materials and methods section but in the presence of 5 mM-NADP and 5 mM-shikimate. Proteolysis was terminated by passing the incubation mixture through a column of soya-bean trypsin inhibitor and then dehydroquinase active sites were labelled by treatment with 3-dehydroquinate and NaB^3H_4. Tube gels (*a*) and (*b*) were run in the presence of sodium dodecyl sulphate and are of untreated *arom* complex and of proteolytically cleaved active-site-labelled *arom* complex respectively. Tube gels (*c*) and (*d*) are polyacrylamide gels run in the presence of 1 M-urea; gel (*c*) was stained for shikimate dehydrogenase activity and gel (*d*) for protein; the three bands seen in these urea gels are numbered 1 to 3 in order of increasing mobility. The first-dimension tube gel for gel (*e*), which is a polyacrylamide slab gel run in the presence of sodium dodecyl sulphate, was a urea gel similar to gels (*c*) and (*d*). The molecular masses of the principal species observed in the presence of sodium dodecyl sulphate are given.

and assuming that the amount of Coomassie Blue stain taken up was proportional both to the size and amount of the labelled polypeptide chains. On this basis the *arom* polypeptide and the polypeptide of M_r 68000 had very similar specific activities, as one would have expected if each contained a single 3-dehydroquinase active site (Table 2).

Polyacrylamide-gel electrophoresis in the presence of 1 M-urea separated three bands of protein, each of which stained for shikimate dehydrogenase activity (Fig. 3, gels *c* and *d*). The polypeptides present in these three bands were identified by second-dimension electrophoresis in the presence of sodium dodecyl sulphate (Fig. 3). This demonstrated that the slowest-moving band in the 1 M-urea gel was uncleaved *arom* polypeptide, the band of intermediate mobility consisted of uncleaved *arom* polypeptide together with both of the major proteolytic fragments, and the fastest-moving band consisted entirely of the fragment of M_r 68000. Since this material of M_r 68000 was derived from a band which stained for shikimate dehydrogenase activity, it follows that the shikimate dehydrogenase active site is also carried on this fragment. Thus the fragment of M_r 68000 contains both the shikimate dehydrogenase and 3-dehydroquinase active sites.

In a further experiment a sample of the trypsin-treated *arom* complex was subjected first to electrophoresis in the presence of 8 M-urea and then to electrophoresis in a second dimension in the presence of sodium dodecyl sulphate. In this case staining a first-dimension gel for shikimate dehydrogenase activity revealed a single activity band which was shown in the second-dimension gel (Fig. 5) to consist entirely of material of M_r 68000. Treatment with 8 M-urea inactivates the shikimate dehydrogenase activity of intact *arom* enzyme complex and of trypsin-treated complex. This activity is not readily recovered by the intact *arom* polypeptide when urea is removed from the gel. In contrast the

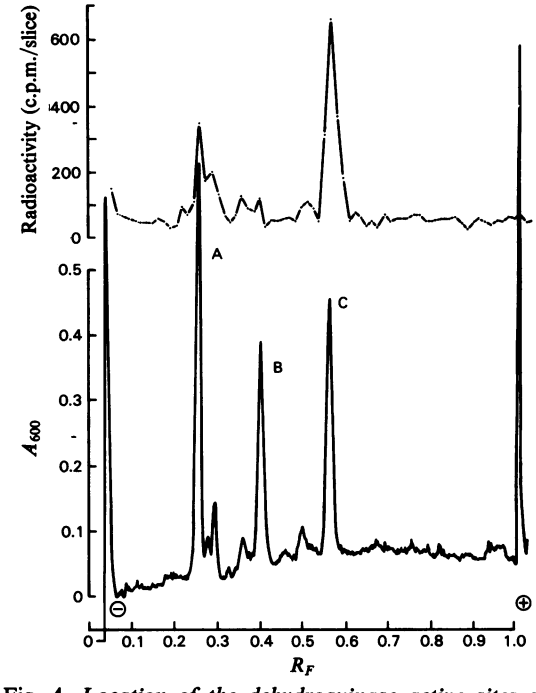


Fig. 4. *Location of the dehydroquinase active sites on fragments of trypsin-treated arom enzyme complex*
Proteolytically cleaved *arom* complex was prepared as described in the legend to Fig. 3 and the 3-dehydroquinase active sites were ^{3}H-labelled as described in the Materials and methods section. The products were separated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The lower trace shows a densitometer scan of the Coomassie Blue-stained gel and the upper trace the radioactivity present in 1 mm discs of a sliced gel similar to the stained gel for which a trace is shown. The principal bands are labelled and have M_r values as follows: A, 165000; B, 110000; C, 68000.

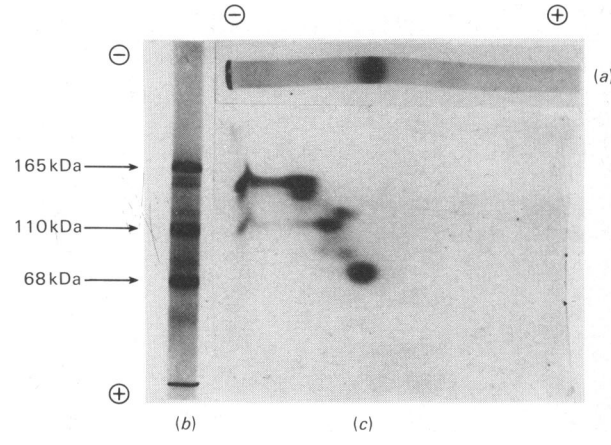


Fig. 5. *Polyacrylamide-gel electrophoresis of trypsin-treated arom enzyme complex in the presence of 8 M-urea*
Proteolysed *arom* complex was prepared as described in the legend to Fig. 3. The products were separated by polyacrylamide-gel electrophoresis either in the presence of 8 M-urea or in the presence of sodium dodecyl sulphate. Gel (*a*) is a urea gel which was subsequently washed free of urea and then stained for shikimate dehydrogenase activity. A duplicate gel also washed free of urea was soaked in sodium dodecyl sulphate-containing buffer and used as the first dimension for the slab gel (*c*), which was also stained for protein. Gels (*b*) and (*c*) were run in the presence of sodium dodecyl sulphate and subsequently stained for protein.

Table 2. *Relative specific radioactivities of the major polypeptides present after trypsin treatment of the arom enzyme complex followed by ^{3}H labelling of the 3-dehydroquinase active sites*
The data presented in the Table are derived from Fig. 4. The relative amount of protein (P) in each gel band was estimated by dividing the peak area by the band M_r. The relative specific radioactivity of each band was then calculated by dividing the radioactivity (R) by the amount of protein (P).

Gel band	Band M_r	Total radioactivity in gel band (R) (c.p.m.)	Peak area (arbitrary units)	$10^5 \times$ Relative amount of protein (P)	Relative specific radioactivity (R/P)	Relative specific radioactivity of band
A	165000	850	3.40	2.06	413	1.00
B	110000	63	1.50	1.37	46	0.11
C	68000	1230	1.96	2.89	426	1.03

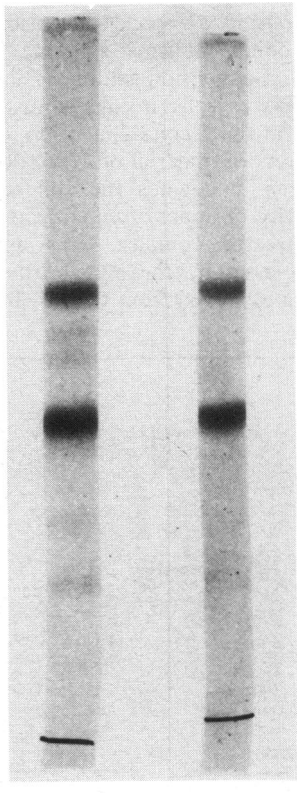


Fig. 6. *Cross-linking of trypsin-treated arom enzyme complex*

A sample of proteolytically cleaved *arom* complex, prepared as described in the legend to Fig. 3 and behaving on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate like that shown in Fig. 3, gel (*a*), was cross-linked with dimethyl suberimidate as described in the Materials and methods section. A control sample of *arom* complex was also cross-linked with dimethyl suberimidate under the same conditions. The gels shown are 3% polyacrylamide gels run in the presence of sodium dodecyl sulphate; gel (*a*) is of cross-linked, trypsin-treated *arom* complex and gel (*b*) of cross-linked *arom* complex.

fragment of the *arom* polypeptide with M_r 68000 regains much of its shikimate dehydrogenase activity when urea is washed from the gel. Since in this experiment the 8 M-urea electrophoresis results in the complete separation of the fragment of M_r 68000 from all the other components, it follows that all the information for the refolding of the shikimate dehydrogenase domain is present within this fragment.

The native *arom* complex is a dimer of identical polypeptide chains (Lumsden & Coggins 1977, 1978) and so it was of considerable interest to know whether the trypsin-treated complex retained this quaternary structure. Simple polyacrylamide-gel electrophoresis of trypsin-treated *arom* complex showed only a single protein band and a single shikimate dehydrogenase activity band, just as the native enzyme does (Lumsden & Coggins, 1977). In contrast, a gel run in the presence of 1 M-urea shows three protein bands and three shikimate dehydrogenase activity bands (Fig. 3, gels *c* and *d*). These observations suggested that the proteolytically cleaved *arom* complex had retained its native quaternary structure but that mild urea treatment caused dissociation of the fragments. Further evidence that the proteolysed *arom* complex had retained its quaternary structure came from cross-linking experiments with dimethyl suberimidate. Samples of intact *arom* complex and trypsin-treated *arom* complex were treated with dimethyl suberimidate at pH 8 and then subjected to polyacrylamide-gel electrophoresis. The proteolysed material, which before cross-linking gave a pattern like gel (*a*) of Fig. 3 now showed only two major bands of M_r 165000 and M_r 330000, just like those seen with the intact *arom* complex (Fig. 6).

The susceptibility of the *arom* complex to other proteinases was also investigated. It was found to be more susceptible to proteolysis by subtilisin than by trypsin. When a final concentration of subtilisin of only 3 μg/ml and a temperature of 0°C (compared with 10 μg/ml and 25°C for the trypsin experiments) were used, the half-life of the shikimate kinase activity was 3.5 min. During this experiment with subtilisin, the 3-dehydroquinase and shikimate dehydrogenase activities were scarcely affected; after 60 min both of these activities remained at 90% of their initial level. Analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a sample of *arom* complex which had been treated with subtilisin for 30 min at 0°C showed that the principal proteolysis product was the polypeptide of M_r 68000 already observed in the experiments with trypsin (Figs. 3 and 5). Substrate labelling of this material with 3-dehydroquinate and NaB^3H_4 showed that most of the radioactivity was associated with the fragment of M_r 68000. Some radioactivity was also associated with a broad protein band found to correspond to a M_r of 52500 and with two faint bands which had M_r values of 109000 and 96000 (Fig. 7).

Proteolysis of the *arom* complex with chymotrypsin was also found to be rapid. With a chymotrypsin concentration of 1.25 μg/ml the half-life of the shikimate kinase activity at 25°C was found to be 7.5 min. As with trypsin and subtilisin the 3-dehydroquinase and shikimate dehydrogenase activities were only very slowly decreased. After

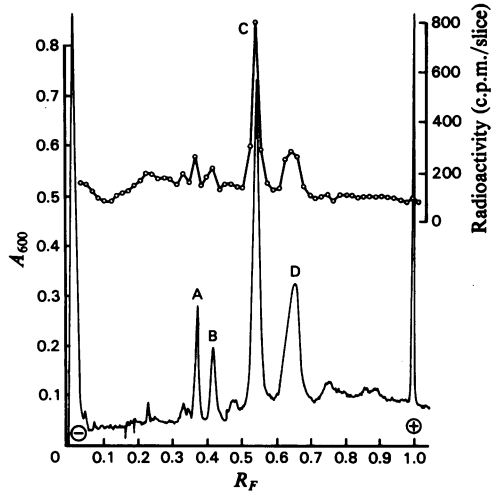


Fig. 7. *Location of dehydroquinase active sites on fragments of subtilisin-treated arom enzyme complex*

Arom enzyme complex was incubated for 30 min at 0°C with subtilisin as described in the Materials and methods section. Proteolysis was terminated with phenylmethanesulphonyl fluoride and then 3-dehydroquinase active sites were ^{3}H-labelled as described in the Materials and methods section. The products were separated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The lower trace shows a densitometer scan of a Coomassie Blue-stained gel and the upper trace the radioactivity present in 1 mm discs of a sliced gel similar to the stained gel for which a trace is shown. The principal bands are labelled and have M_r values as follows: A, 109000; B, 96000; C, 68000; D, 52500.

30 min both these activities were still at 90% of their initial levels. The two major products of proteolysis at this stage had M_r values of 68000 and 51000.

The *arom* polypeptide was found to be very resistant to proteolysis by elastase. After 1 h of treatment with 5 μg of elastase/ml at 25°C sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that less than 5% of the *arom* polypeptide had been cleaved. In contrast, after treatment of the complex with papain (5 μg/ml) at 25°C for 5 min, no intact *arom* polypeptide remained. After 30 min treatment with papain the two major products of proteolysis had M_r values of 68000 and 51000.

Discussion

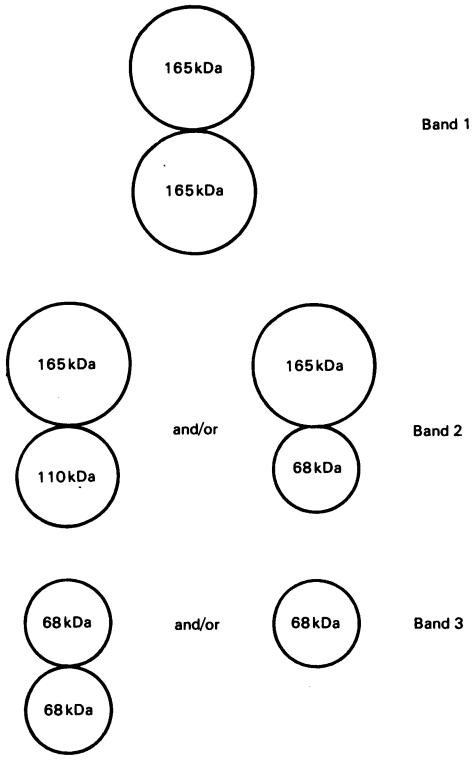
The results presented here clearly indicate that the shikimate dehydrogenase and 3-dehydroquinase activities of the *arom* enzyme complex occur on a contiguous fragment of the *arom* polypeptide of M_r 68000. This fragment is produced most readily by treatment of the *arom* complex either with trypsin (Figs. 3 and 4) or with subtilisin (Fig. 7). The proteolytic cleavage resulting in formation of the fragment is accompanied by loss of the shikimate kinase activity of the enzyme complex and presumably occurs in a region of the polypeptide essential for this activity (Table 1). The fragment of M_r 68000 is also a major product when the *arom* enzyme complex is treated with chymotrypsin and papain. The fact that trypsin, subtilisin, chymotrypsin and papain can all cleave the *arom* polypeptide in the same region to give this fragment suggests that in the native *arom* enzyme complex there is a short exposed loop of polypeptide chain which is very susceptible to cleavage by these four enzymes. It is noteworthy that this exposed loop is not cleaved by elastase.

Under the conditions described, the fragment of M_r 68000 is resistant to further proteolysis and must therefore consist of a tightly folded globular structure or domain. This bifunctional domain contains the active sites for both shikimate dehydrogenase and 3-dehydroquinase. Chemical-modification evidence indicates that the two active sites are spatially distinct (Smith, 1980; Coggins, 1982), but as yet it has not proved possible to isolate a monofunctional domain carrying either of these activities. Further structural studies on the complex will be needed in order to determine whether the individual enzyme functions are actually associated with much smaller structural domains; for example, the shikimate dehydrogenase activity might be associated with one or two nucleotide-binding domains (Rossmann *et al.*, 1975) and a substrate-binding domain.

Limited proteolysis does not appear to disrupt the quaternary structure of the *arom* enzyme complex. Even when a substantial fraction of the *arom* polypeptide has been cleaved into the fragments of M_r 68000 and 110000 (Fig. 3, gel *b*) the electrophoretic mobility of the complex under non-denaturing conditions is unchanged and cross-linking with dimethyl suberimidate still yields as much cross-linked dimer as cross-linking of the uncleaved complex (Fig. 6). These results, together with the essentially complete retention of the two enzymic activities, indicate that proteolysis is not perturbing the native structure of the *arom* enzyme complex.

The data presented here do not allow a unique interpretation for the structures of the three species separated by electrophoresis in the presence of 1 M-urea (Fig. 3). Some possible interpretations are given in Scheme 2.

Case *et al.* (1969) have presented a very detailed genetic map of the *arom* gene cluster of *N. crassa*. When this map was published it was not realized



Scheme 2. *Proposed structure of the components of the trypsin-treated arom enzyme complex separated by polyacrylamide-gel electrophoresis in the presence of 1 M-urea*

This Figure offers some possible interpretations of the results shown in Fig. 3. The bands are labelled as in Fig. 3 and the numbers within the circles refer to the molecular masses of the various polypeptides.

that the *arom* polypeptide consisted of a single pentafunctional polypeptide chain. The cluster was thought to consist of five separate genes each coding for a different polypeptide chain. It is now clear that the cluster should be considered a single gene coding for a single pentafunctional polypeptide chain (Fincham *et al.*, 1979). The original map can now be interpreted to give the order of enzyme active sites along the polypeptide chain (Scheme 3). Most of the classes of pleiotropic mutants described by Case *et al.* (1969) fit into this scheme. For example, these workers isolated 41 mutants lacking both the 3-dehydroquinase and the shikimate dehydrogenase activities. The revised model of the gene cluster (Scheme 3) places these two activities together at the *C*-terminal end of the *arom* polypeptide, and these mutants accordingly must lack the *C*-terminal region of the *arom* polypeptide. These genetic studies indicate that the fragment of the *arom* polypeptide

Polypeptide chain	*N*-terminus —— *C*-terminus				
Order of enzymes	1	5	4	2	3

Scheme 3. *Model of the structure of the arom gene cluster and the arom polypeptide*

This model is based on the genetic studies of Rines *et al.* (1969) and the limited proteolysis studies reported in the present paper. The enzymes are numbered as in Scheme 1.

with M_r 68000 described here must be derived from the *C*-terminal region of the polypeptide chain. The isolation of the two activities on a single contiguous region of the polypeptide chain is entirely consistent with, and confirms, the genetic map.

Fincham *et al.* (1979) have suggested that the five different functions of the *arom* polypeptide may be associated with separate, semi-independent folding domains. Our results clearly demonstrate that the two enzyme activities associated with the *C*-terminal region of the arom polypeptide do not require the intact polypeptide chain for activity. The shikimate dehydrogenase activity, in particular, exists as an independent functional domain which can refold and regain enzyme activity after denaturation with urea, even in the absence of the rest of the polypeptide chain.

The five enzyme activities which in *Neurospora crassa* occur on the pentafunctional *arom* polypeptide chain are very differently organized in some other organisms. In *Escherichia coli* the genes coding for the five enzyme activities are separately located (Bachmann & Low, 1980) and there is no evidence of physical association (Berlyn & Giles, 1969). In a number of yeasts (Bode & Birnbaum, 1981) and fungi (Ahmed & Giles, 1969) the five enzymes occur as a multienzyme complex, although no evidence concerning the number and kind of subunits is available. In some other yeasts (Bode & Birnbaum, 1981) two of the five enzyme activities, shikimate dehydrogenase and 3-dehydroquinase, occur as a multienzyme complex, whereas the other three enzymes are separable. In *Euglena gracilis* there is a pentafunctional polypeptide (Patel & Giles, 1979), whereas in the moss *Physcomitrella patens* two of the five enzyme activities, shikimate dehydrogenase and 3-dehydroquinase, occur on a bifunctional polypeptide which does not associate with the other three activities (Polley, 1978). The association of shikimate dehydrogenase with 3-dehydroquinase seems to occur widely in plants. Where these two enzymes have been studied, they seem to co-purify (Boudet *et al.*, 1975; Koshiba, 1978, 1979). It is therefore of some interest that these two enzyme activities occur next to each other on the *Neurospora*

arom polypeptide and can be isolated on a bifunctional fragment of M_r 68000. This is significantly larger than the M_r value of 48000 found for the bifunctional polypeptide of *P. patens*, but it is possible that not all of the fragment of the Neurospora *arom* polypeptide with M_r 68000 is required for the two enzyme functions.

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References

Ahmed, S. I. & Giles, N. H. (1969) *J. Bacteriol.* **99**, 231–237

Bachmann, B. J. & Low, K. B. (1980) *Microbiol. Rev.* **44**, 1–56

Bates, D. L., Harrison, R. A. & Perham, R. N. (1975) *FEBS Lett.* **60**, 427–430

Berlyn, M. B. & Giles, N. H. (1969) *J. Bacteriol.* **99**, 222–230

Bode, R. & Birnbaum, D. (1981) *Z. Allg. Mikrobiol.* **21**, 417–422

Boudet, A. M., Lecussan, R. & Boudet, A. (1975) *Planta* **124**, 67–75

Butler, J., Alworth, W. & Nugent, M. (1974) *J. Am. Chem. Soc.* **96**, 1617–1618

Case, M. E., Burgoyne, L. & Giles, N. H. (1969) *Genetics* **63**, 581–588

Coggins, J. R., Lumsden, J. & Malcolm, A. D. B. (1977) *Biochemistry* **16**, 1111–1116

Coggins, J. R. (1982) *Neurospora Newsl.* **29**, 8

Danson, M. J., Hale, G., Johnson, P., Perham, R. N., Smith, J. & Spragg, P. (1979) *J. Mol. Biol.* **129**, 603–17

Dautry-Varsat, A. & Garel, J.-R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5979–5982

Dileepan, K. N., Lin, C. Y. & Smith, S. (1978) *Biochem. J.* **175**, 199–206

Erlanger, B. F., Kokowsky, N. & Cohen, J. W. (1961) *Arch. Biochem. Biophys.* **95**, 271–278

Fincham, J. R. S., Day, P. R. & Radford, A. (1979) *Fungal Genetics*, 4th edn., Blackwell Scientific Publications, Oxford

Gaertner, F. H. & Cole, K. W. (1977) *Biochem. Biophys. Res. Commun.* **75**, 259–264

Giles, N. H., Case, M. E., Partridge, C. W. H. & Ahmed, S. I. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1453–1460

Kirschner, K. & Bisswanger, H. (1976) *Annu. Rev. Biochem.* **45**, 143–166

Koshiba, T. (1978) *Biochim. Biophys. Acta* **522**, 10–18

Koshiba, T. (1979) *Plant Cell Physiol.* **20**, 667–670

Lumsden, J. & Coggins, J. R. (1977) *Biochem. J.* **161**, 599–607

Lumsden, J. & Coggins, J. R. (1978) *Biochem. J.* **169**, 441–444

McCarthy, A. D. & Hardie, D. G. (1983) *Eur. J. Biochem.* **130**, 185–193

Patel, V. B. & Giles, N. H. (1979) *Biochim. Biophys. Acta* **567**, 24–34

Polley, L. D. (1978) *Biochim. Biophys. Acta* **526**, 259–266

Rines, H. W., Case, M. E. & Giles, N. H. (1969) *Genetics* **61**, 789–800

Rossmann, M. A., Liljas, A., Branden, C. & Banaszak, L. (1975) *Enzymes 3rd Ed.* **11**, 62–102

Ryan, L. D. & Vestling, C. S. (1974) *Arch. Biochem. Biophys.* **160**, 279–284

Smith, D. D. S. (1980) Ph.D. Thesis, University of Glasgow

Tan, L. U. L. & MacKenzie, R. E. (1979) *Can. J. Biochem.* **57**, 806–812

Vitto, A. & Gaertner, F. H. (1978) *Biochem. Biophys. Res. Commun.* **82**, 977–981