Biochem. J. (1978) **169**, 441–444
Printed in Great Britain

The Subunit Structure of the *arom* Multienzyme Complex of *Neurospora crassa*

EVIDENCE FROM PEPTIDE 'MAPS' FOR THE IDENTITY OF THE SUBUNITS

By JAMES LUMSDEN and JOHN R. COGGINS
Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

(*Received* 2 *November* 1977)

Evidence was obtained, from polyacrylamide-gel electrophoresis in the presence of urea and from peptide 'mapping' of specifically labelled cysteine- and methionine-containing peptides, that the two subunits of the *arom* multienzyme complex of *Neurospora crassa* are chemically very similar and possibly identical.

Five steps on the early common pathway of aromatic amino acid biosynthesis in *Neurospora crassa* are catalysed by a multienzyme complex (Giles *et al.*, 1967). If this *arom* multienzyme complex is purified rapidly in the presence of proteinase inhibitors, it is found to be composed of two subunits of mol.wt. 165000 (Lumsden & Coggins, 1977; Gaertner & Cole, 1977). The genes that code for the five enzyme activities are clustered and for two of the activities allelic complementation has been observed (Giles *et al.*, 1967). The genes for these two enzymes are located one at each end of the *arom* cluster, which is believed to be transcribed as a single mRNA molecule (Giles *et al.*, 1967; Jacobson *et al.*, 1972). These data strongly suggest the *arom* enzyme complex consists of a dimer of identical pentafunctional polypeptide chains (Lumsden & Coggins, 1977). To obtain chemical evidence for the identity of the subunits we have determined the amino acid composition of the *arom* enzyme complex and 'mapped' its cysteine- and methionine-containing peptides.

Materials and Methods

Chemicals and other materials were obtained either from the sources described in the appropriate references or from BDH Chemicals, Poole, Dorset, U.K., except for the following: methanesulphonic acid, tryptamine and *N*-ethylmorpholine were from Koch–Light, Colnbrook Bucks., SL3 0BZ, U.K.; all radioactive compounds were from The Radiochemical Centre, Amersham, Bucks., U.K.; proteinases were from Worthington Biochemical Corp., Freehold, NJ 07728, U.S.A., except carboxypeptidase C, which was kindly donated by Dr. J. Kay, Department of Biochemistry, University College, Cardiff CF1 1XL, Wales, U.K.; dithiothreitol, N^{α}-benzoyl-L-arginine and hen egg-white lysozyme were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; Fuji RX X-ray film was from Fuji Products, X-ray Division, Hanimex U.K., Dorcan, Swindon, Wilts., U.K.

The *arom* enzyme complex was prepared as described previously (Lumsden & Coggins, 1977) except that enzyme to be used for *C*-terminal analyses was extracted in the presence of 3mM-hydrocinnamic acid and 1mM-benzoyl-L-arginine in an attempt to inhibit endogenous carboxypeptidases (Terai *et al.*, 1976). Purified enzyme was dialysed against excess 0.5% (w/v) NH_4HCO_3, freeze-dried and stored at −20°C.

Gel electrophoresis

Electrophoresis in 3% (w/v) polyacrylamide gels in the presence of 8M-urea was by the method of Davis (1964) as modified by Hayes & Wellner (1969). Protein bands were stained as described previously (Lumsden & Coggins, 1977).

End-group analyses

N-Terminal amino acid analyses were performed as described by Woods & Wang (1967) and Gray (1972). *C*-Terminal amino acid residues were investigated by partial digestion with carboxypeptidases, with a micro-modification of the method of Ambler (1972); 250pmol of [^{14}C]carboxymethylated *arom* enzyme complex was dissolved in 50μl of 0.2M-*N*-ethylmorpholine acetate (pH 8.5)/56mM-sodium dodecyl sulphate and incubated at 37°C for up to 5h with 25pmol of carboxypeptidase A or carboxypeptidase B or a mixture of the two. At the end of this period, 20μl of a mixture of standard ^{14}C-labelled amino acids (Brown & Perham, 1973) was added and the reaction was terminated by the addition of 10mg of Dowex 50W-X8 resin (H^+ form; 20–50 mesh). The amino acids were recovered as described (Ambler, 1972) and quantified by using the double-isotope-

labelling method of Brown & Perham (1973). In all cases a parallel digestion of 250pmol of lysozyme (*C*-terminal sequence -Arg-Leu) was carried out as a test of the validity of the method.

Amino acid analyses

Samples of performic acid-oxidized *arom* enzyme complex (Hirs, 1967) were hydrolysed and analysed by the method of Spackman *et al.* (1958). Tryptophan and tyrosine were determined both spectrophotometrically (Edelhoch, 1967) and after hydrolysis of carboxymethylated *arom* enzyme complex with methanesulphonic acid (Moore, 1972). The specific absorption coefficient of a sample of the *arom* protein dissolved in 0.5% NH_4HCO_3 (pH8.5) was also determined by amino acid analysis after hydrolysis with HCl. Norleucine was used as an internal standard for this determination and for all the amino acid analyses.

Carboxymethylation of the arom enzyme complex

(*a*) *Cysteine residues* (*Gibbons & Perham*, 1970). *arom* enzyme complex (1mg) was dissolved in 0.25ml of 0.1M-Tris/HCl (pH8.2) / 8M-urea / 2mM-dithiothreitol and incubated for 1h in the dark under an atmosphere of N_2 at room temperature (20°C). The solution was then made 15mM in iodo[^{14}C]acetic acid (13.4Ci/mol) and incubated for a further 1h. The reaction was terminated by the addition of dithiothreitol to a final concentration of 30mM and the carboxymethylated protein was recovered by gel filtration through a 10ml column of Sephadex G-25 (fine grade) equilibrated with 10% (v/v) formic acid. The final specific radioactivity in two separate experiments was 1.1μCi/mg, which corresponds to the calculated value for quantitative conversion.

(*b*) *Methionine residues* (*Vithaythil & Richards*, 1960). Carboxymethylated protein (0.5mg), prepared as above by using unlabelled reagent, was dissolved in 0.2ml of 5% (v/v) formic acid containing 60mM-iodo[^{14}C]acetic acid (4.2Ci/mol) and incubated in the dark for 40h at 37°C. The labelled protein was recovered by gel filtration as before. In two separate experiments the specific radioactivity (0.8μCi/mg) corresponded to greater than 95% conversion.

Peptide 'mapping'

When the *arom* enzyme complex was treated with trypsin and chymotrypsin, together or separately, insoluble cores unsuitable for peptide 'mapping' were obtained. This problem was solved by the prior use of pepsin by a method similar to that of Kaplan *et al.* (1971); 2nmol of [^{14}C]carboxymethylated *arom* enzyme complex in 10% (v/v) formic acid was digested with pepsin (2%, w/w) for 16h at 37°C. The digest was then adjusted to pH8.2 by careful addition of 4M-NH_3, and trypsin (0.5%, w/w) was added. After 4h at 37°C, a second, equal, addition of trypsin was made and the incubation continued for a further 4h. Finally, chymotrypsin (1%, w/w) was added and digestion left to proceed for 12h at 37°C. The resulting peptic/tryptic/chymotryptic digest was diluted with 25vol. of water and freeze-dried repeatedly to remove all traces of ammonium formate. The residue was dissolved in a few microlitres of 5% (v/v) formic acid for spotting on to silica-gel thin layers and peptide 'maps' were prepared as described by Bates *et al.* (1975). ^{14}C-labelled peptides were detected by radioautography with Fuji RX X-ray film.

Results and Discussion

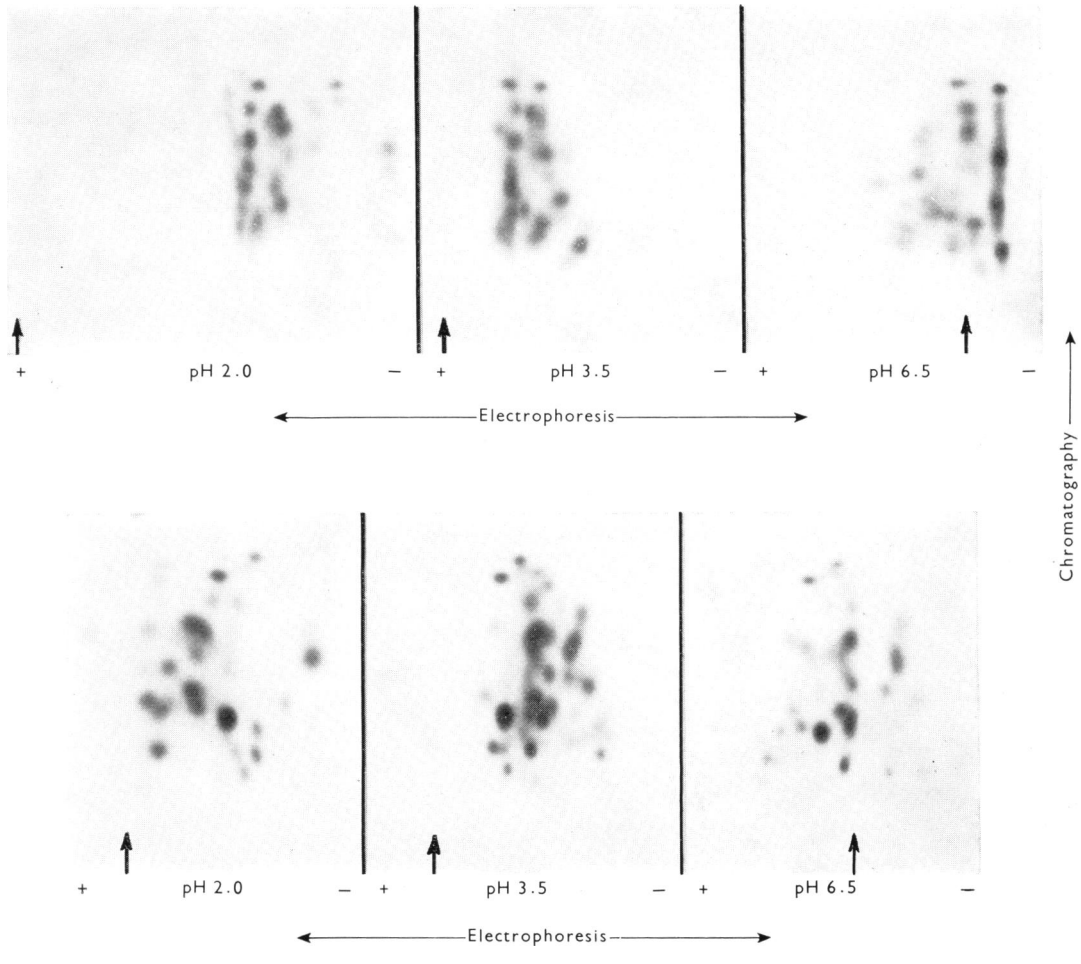
Urea gels

A single protein band was observed on polyacrylamide-gel electrophoresis of carboxymethylated *arom* enzyme complex in the presence of 8M-urea. This suggests that the subunits were identical with respect to charge. An earlier study (Lumsden & Coggins, 1977) had established that they are identical with respect to size.

Amino acid composition

The amino acid composition of the *arom* enzyme complex is presented in Table 1. The results have been calculated on the basis of the subunit mol.wt. of 165000 reported earlier (Lumsden & Coggins, 1977). Our amino acid composition is different from that reported by Burgoyne *et al.* (1969) (Table 1) for *arom* enzyme complex that was almost certainly proteolytically damaged (Lumsden & Coggins, 1977; Gaertner & Cole, 1976, 1977). The differences may be due to the loss of some parts of the *arom* polypeptide during the earlier purification procedure.

One feature of the amino acid composition requires comment. The tryptophan content determined by amino acid analysis was 14mol/165000g of protein, whereas the spectrophotometric method of Edelhoch (1967) gave a value of 19mol/165000g of protein. Since the recovery of tryptophan in control experiments with methanesulphonic acid was in the range 94–98% it is difficult to attribute this discrepancy to simple destruction of tryptophan during the acid hydrolysis. The specific absorption coefficient ($A^{1\%}_{1cm}$) determined by amino acid analysis for the native *arom* protein is 11.0, whereas the $A^{1\%}_{1cm}$ calculated from the tryptophan and tyrosine content found after hydrolysis is only 8.0. Although this may be due partly to a hyperchromic effect associated with burying aromatic side chains, it also suggests that the tryptophan content measured by amino acid analysis may be too low.



EXPLANATION OF PLATE 1

Radioautographs of peptide 'maps' of peptic/tryptic/chymotryptic digests of [^{14}C]*carboxymethylated arom multienzyme complex*
(*a*) Cysteine-containing peptides labelled by the method of Gibbons & Perham (1970); (*b*) methionine-containing peptides labelled by the method of Vithayathil & Richards (1960). The 'maps' were prepared as described by Bates *et al.* (1975) (see the Materials and Methods section). Samples of 0.25 nmol of labelled protein were used for each 'map'; radioautography was for 1–2 days (cysteine 'maps') or 6–9 days (methionine 'maps'). The arrow indicates the origin. Electrophoresis was performed at the pH values shown.

Table 1. *Amino acid composition of the arom multienzyme complex*

The values represent the means of three values determined after hydrolysis of performic acid-oxidized protein with 6M-HCl at 105°C for 24, 48 and 96h except where indicated in the footnotes.

	Composition (mol/165000g of protein)	
Amino acid	The present work	Burgoyne *et al.* (1969)
Trp*	14 (19¶)	22
Lys	80	56
His	33	22
Arg	78	56
Cys†	14	12
Asx	136	157
Met‡	32	26
Thr§	86	86
Ser§	103	106
Glx	165	164
Pro	86	94
Gly	108	121
Ala	151	170
Val‖	121	106
Ile‖	82	83
Leu	135	186
Tyr*	42 (39¶)	34
Phe	49	38

* Determined after hydrolysis of reduced carboxymethylated protein with 4M-methanesulphonic acid for 48h at 105°C.

† Determined as cysteic acid.

‡ Determined as methionine sulphone.

§ Values obtained by extrapolation to zero time.

‖ Values for 96h hydrolysis only.

¶ Values in parentheses were determined by the spectrophotometric method of Edelhoch (1967).

Peptide 'maps'

The amino acid composition indicates that 1mol of the *arom* dimer (mol.wt. 330000) contains 28mol of cysteine and 68mol of methionine. If the subunits of the *arom* enzyme complex were identical one would expect the 'maps' to show approx. 14 cysteine- and 34 methionine-containing peptides. The peptide 'maps', obtained by electrophoresis at three different pH values, but with a common chromatographic procedure (Plate 1), showed the presence of approx. 16–19 cysteine- and 28–32 methionine-containing peptides. This interpretation is based on visual comparison of the 'maps' obtained at the three different pH values; it establishes that the two polypeptide chains are at least substantially homologous and possibly even identical.

A number of very faint spots, which appeared only on prolonged radioautography, are probably due to the comparatively broad specificity of action of pepsin, which may have resulted in more than one cleavage pattern from certain sequences. There were a small number of particularly intense spots, which were not further resolved at any of the pH values tested. These may be due to the presence of more than one labelled residue in the peptide or they may be due to the presence of more than one equivalent of a particular peptide and thus be indicative of a certain amount of internal sequence homology within the *arom* polypeptide (cf. Waterson & Konigsberg, 1974). This might be expected of five enzymes, which, as they catalyse consecutive reactions of a biosynthetic pathway, must recognize structurally similar substrates.

End-group analyses

Further evidence for the chemical identity of the subunits was sought by end-group analysis. No *N*-terminal amino acid could be detected by reaction with dansyl chloride. Hydrolysis times of 18h and 4h to minimize any possible destruction of dansylproline) were used, but only spots due to N^{ε}-dansyllysine and *O*-dansyltyrosine were observed. Brown & Roberts (1976) have reported that a large percentage of *N. crassa* proteins are N^{α}-acetylated and it seems likely that the *N*-terminus of the *arom* polypeptide is similarly blocked.

Most published methods of determining *C*-terminal amino acids require at least 20nmol of protein (see Ambler, 1972). In the present work by using the very sensitive amino acid analysis method of Brown & Perham (1973) we have developed a method for *C*-terminal analysis requiring only 250pmol of protein. However, although the method was found to be completely satisfactory for the test protein, lysozyme, no *C*-terminal amino acid could be identified for the *arom* enzyme complex despite prolonged incubation with carboxypeptidases A and/or B. *C*-Terminal amides are common among the peptide hormones, but there are no known examples of enzymes that have a blocked *C*-terminus (Croft, 1973, 1974, 1976). One possible explanation for our failure to detect a *C*-terminus is that the large *arom* polypeptides, even in the presence of dodecyl sulphate, retain sufficient tertiary structure to keep their *C*-terminal residues buried or otherwise inaccessible to carboxypeptidase attack. A second possibility is that the *C*-terminal sequence is resistant to enzymic hydrolysis; this might be expected for a terminal or penultimate proline residue. Unfortunately it was not possible to check for the presence of proline by using carboxypeptidase C (Hayashi, 1976), since we were unable to find a solvent in which carboxymethylated *arom* enzyme complex was soluble and in which carboxypeptidase C remained active.

Conclusion

The data presented in the present paper establish that the two subunits of the *arom* multienzyme complex have very similar chemical sequences. This information was obtained from experiments carried out on an unusually small scale; the enzyme digests were performed on 2nmol of protein and the end-group analyses and the peptide 'maps' on 0.25nmol. Final proof that the two subunits are identical will require total sequence analysis; this will require much larger amounts of the *arom* enzyme complex than are presently available.

We thank Mr. John Greene for growing the cells, Mr. Robert Blackie for performing the amino acid analyses and the Science Research Council for a grant to J. R. C. (B/RG/6927.9) and for a fellowship (J. L.).

References

Ambler, R. P. (1972) *Methods Enzymol.* **25**, 143–154
Bates, D. L., Perham, R. N. & Coggins, J. R. (1975) *Anal. Biochem.* **68**, 175–184
Brown, J. L. & Roberts, W. K. (1976) *J. Biol. Chem.* **251**, 1009–1014
Brown, J. P. & Perham, R. N. (1973) *Eur. J. Biochem.* **39**, 69–73
Burgoyne, L., Case, M. E. & Giles, N. H. (1969) *Biochim. Biophys. Acta* **191**, 452–462
Croft, L. R. (1973) *Handbook of Protein Sequences*, Joynson–Bruvvers, Eynsham
Croft, L. R. (1974) *Handbook of Protein Sequences: Suppl. A*, Joynson–Bruvvers, Eynsham
Croft, L. R. (1976) *Handbook of Protein Sequences: Suppl. B*, Joynson–Bruvvers, Eynsham
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Edelhoch, H. (1967) *Biochemistry* **6**, 1948–1954
Gaertner, F. H. & Cole, K. W. (1976) *Arch. Biochem. Biophys.* **177**, 566–573
Gaertner, F. H. & Cole, K. W. (1977) *Biochem. Biophys. Res. Commun.* **75**, 259–264
Gibbons, I. & Perham, R. N. (1970) *Biochem. J.* **116**, 843–849
Giles, N. H., Case, M. E., Partridge, C. W. H. & Ahmed, S. I. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 1453–1460
Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–142
Hayashi, R. (1976) *Methods Enzymol.* **45**, 568–587
Hayes, M. B. & Wellner, D. (1969) *J. Biol. Chem.* **244**, 6636–6644
Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197–199
Jacobson, J. W., Hart, B. A., Doy, C. H. & Giles, N. H. (1972) *Biochim. Biophys. Acta* **289**, 1–12
Kaplan, H., Stevenson, K. J. & Hartley, B. S. (1971) *Biochem. J.* **124**, 289–299
Lumsden, J. & Coggins, J. R. (1977) *Biochem. J.* **161**, 599–607
Moore, S. (1972) in *Chemistry and Biology of Peptides* (Meienhofer, J., ed,), pp. 629–653, Ann Arbor Science Publishers, Ann Arbor
Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
Terai, M., Furihata, C., Matsushima, T. & Sugimura, T. (1976) *Arch. Biochem. Biophys.* **176**, 621–629
Vithayathil, P. J. & Richards, F. M. (1960) *J. Biol. Chem.* **235**, 2343–2351
Waterson, R. M. & Konigsberg, W. H. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 376–380
Woods, K. R. & Wang, K.-T. (1967) *Biochim. Biophys. Acta* **133**, 369–370