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ISOENZYMES AND ALLOSTERIC INHIBITION OF *NEUROSPORA CRASSA* 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHASE

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SUMMARY

1. The molecular sieving on agarose of the 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15) (DAHP synthase) from the fungus *Neurospora crassa* is described. Isoenzymes inhibited by the allosteric ligands, phenylalanine, tyrosine, and tryptophan (DAHP synthases (Phe), (Tyr) and (Trp)) were separated. The substrate phosphoenolpyruvate stabilizes all forms of DAHP synthases (Tyr) and (Phe) and is necessary for their recovery.

2. The influence of all possible combinations of the three allosteric ligands on the apparent molecular weight of isoenzymes was determined in Tris–maleate and phosphate buffers (pH 7.4). It is concluded that DAHP synthases (Phe) and (Tyr) dissociate to half-molecules in the presence of their specific allosteric ligands but that DAHP synthase (Trp) does not. Non-inhibited activity was associated with the inhibited isoenzymes rather than existing independently.

3. The non-inhibited DAHP synthases derived from DAHP synthases (Phe) and (Tyr) by mutation (allosteric inhibition-negative mutants) did not dissociate to half-molecules in the presence of the parental allosteric inhibitor. It is concluded that dissociation of the wild-type isoenzymes is part of the normal mechanism of allosteric inhibition.

4. Non-exclusive binding of the substrate phosphoenolpyruvate and the allosteric ligands phenylalanine and tyrosine is proposed.

5. Those features of the *Neurospora crassa* enzyme presently regarded as unique by comparison with *Escherichia coli* enzyme are summarized.

Abbreviations: DAHP synthase, 3-deoxy-L-*arabino*-heptulosonate 7-phosphate synthase; DAHP synthase (Phe), (Tyr) and (Trp), isoenzymes inhibited by the allosteric ligands, phenylalanine, tyrosine and tryptophan, respectively.

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INTRODUCTION

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15) (DAHP synthase) is the first enzyme specific to the shikimate pathway of aromatic biosynthesis in microorganisms[1]. The *Neurospora crassa* enzyme is inhibited by three allosteric ligands, tyrosine, phenylalanine and tryptophan, the major end products of the pathway[1–5]. A normal wild-type extract is about 40–45% inhibited by phenylalanine, similarly by tyrosine and only about 10–15% by tryptophan. However, the instability of the phenylalanine- and tyrosine-inhibited portions during the usual processes of manipulation and purification lead to preparations 90–100% inhibited by tryptophan. Investigation of the enzyme under such conditions result in unbalanced and inconclusive assessments[5].

The present paper provides an approach which leads to the identification of isoenzymes designated DAHP synthases (Phe), (Tyr) and (Trp) to indicate the inhibiting allosteric ligand. It will be shown that DAHP synthases (Phe) and (Tyr) each exist in at least two forms of which one is favored by a low concentration of the allosteric ligand. This property is eliminated in allosteric-inhibition negative mutants showing that the change in molecular form is part of the normal mechanism of allosteric inhibition.

A biochemical–genetic analysis establishing that DAHP synthases (Phe), (Tyr) and (Trp) are encoded by three distinct genes, with in each case a high degree of probability that the polypeptide concerned is essential not only for activity but also allosteric inhibition, has already been published[6]. Preliminary references to wild-type isoenzymes have been made in a number of publications[1–4,6].

MATERIALS AND METHODS

Organisms

The wild type is *Neurospora crassa* 74A (DeSerres, Oak Ridge, also used at Yale for investigations of aromatic biosynthesis). The isolation and properties of the three mutant strains containing DAHP synthase activity-negative and allosteric inhibition-negative mutations (*arom-6* (DH1) *arom-7*$^{r(DH25)}$ *arom-8* (DH8); *arom-6*$^{r(DH22)}$ *arom-7* (DH7) *arom-8* (DH8) and *arom-6* (DH1) *arom-7* (DH7) *arom-8*$^{r(DH19)}$) were described by HALSALL AND DOY[6].

Culture

Conditions of culturing were described previously[4,6]. All strains were grown on minimal medium.

Extracts

The general procedure for the preparation of extracts was described previously[4]. In the present experiments mycelia were stored for some time at −10 to −15° before use. The data of Figs. 1 and 2 derive from the same batch of 74A mycelium but different extracts. A large batch of 74A mycelium was used to obtain all other wild-type data. Extracts were ground in the mixtures used for subsequent elution from agarose columns.

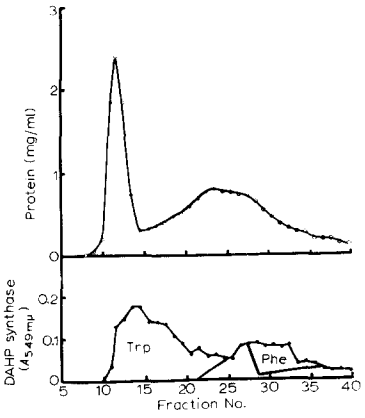


Fig. 1. Molecular sieving of *N. crassa* 74A DAHP synthase in the absence of phosphoenolpyruvate. ○—○, protein, the sharp peak marks the front (Fraction 12) and the protein elution pattern was similar for all Neurospora extracts used in this paper; ●—●, total DAHP synthase activity, the portions inhibited by tryptophan and phenylalanine are indicated (Trp and Phe). Extract prepared in and eluted with 0.05 M KH_2PO_4–NaOH buffer (pH 6.4) containing dithiothreitol and $MgSO_4$ (each 0.1 mM) other details see MATERIALS AND METHODS. Dithiothreitol and $MgSO_4$ were omitted in later experiments (see ref. 6).

Molecular sieving

Crude extracts (10 ml) were applied to columns calibrated with proteins of known molecular weight. Reference should be made to HALSALL AND DOY[6] for details including an example of the elution profile of a single molecular species (bovine serum albumin monomer).

The data of Figs. 1 and 2 were obtained with an agarose (Bio-Gel A-0.5 m, 100–200 mesh) column approx. 2.6 cm × 93 cm and of all other figures, approx. 2.5 cm × 90 cm. With the exception of the experiments of Figs. 1 and 2, a Bio-Gel P-2 column (200–400 mesh, exclusion limit mol. wt. 1600) approx. 1.5 cm × 22 cm was

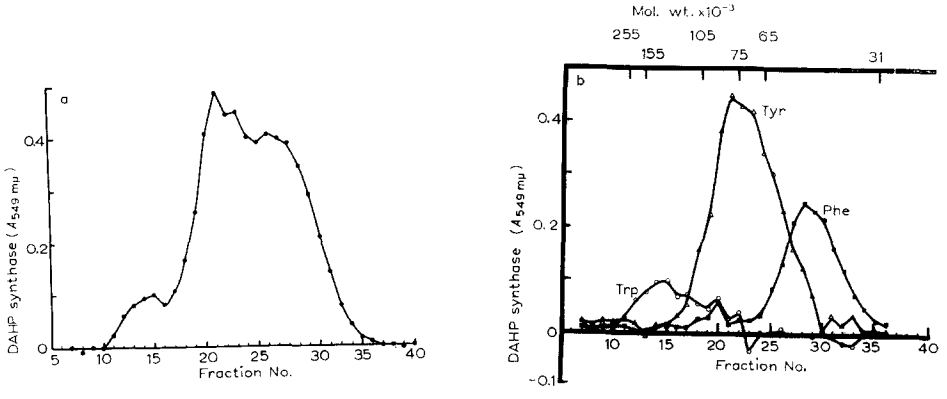


Fig. 2. Molecular sieving of DAHP synthase in presence of 0.1 mM phosphoenolpyruvate; other details as for Fig. 1. a. ●—●, total activity. b. Distribution of portions inhibited by tryptophan (DAHP synthase (Trp)) (○—○); tyrosine (DAHP synthase (Tyr)) (△—△); and phenylalanine (DAHP synthase (Phe)) (■—■). The molecular weight calibration is provided to indicate the sieving range of the column not molecular weight of DAHP synthase isoenzymes (see text).

placed in series before the agarose. The entire system was equilibrated (5 column vols.) with the mixtures used for the preparation of extracts and elution. Elution was at 60 ml/h with 5.7–5.9-ml fractions at 2–5°.

DAHP synthase

DAHP synthase was assayed essentially as described previously[4]. The data of Figs. 3–7 were obtained using 0.025-ml samples of fractions, phosphoenolpyruvate and erythrose 4-phosphate (each 0.25 mM), and as required, phenylalanine, tyrosine or tryptophan (50 μM) in a total volume of 0.25 ml. Activity ($A_{549\ m\mu}$) was read on a Gilford 300 spectrophotometer with digital read out, accuracy $\pm A_{549\ m\mu} = 0.01$. Inhibited activity represents the difference between assays with and without the allosteric ligand concerned.

Protein

Protein was estimated by the method of LOWRY *et al.*[7].

RESULTS

The following describes the molecular sieving of crude extracts of *N. crassa* wild-type 74A on agarose under a wide variety of conditions. This method was selected originally because it allowed the fast flow rates essential for the rapid analysis of an unstable enzyme predicted to exist as isoenzymes. It later proved a particularly good choice because separation is largely dependent on differences in molecular weight and conformation.

The recognition of DAHP synthase isoenzymes

The presence of the substrate phosphoenolpyruvate is essential for good recovery of two of the three classes of allosterically inhibited isoenzymes. Without column equilibration and elution with phosphoenolpyruvate, only 10–15% of starting activity was recovered and most of that was inhibited by tryptophan (Fig. 1). Traces of phenylalanine-inhibited activity were found if fractions were analyzed immediately after collection. Equilibration and elution with buffered phosphoenolpyruvate (0.1 mM) resulted in about 85% recovery of the initial activity (compare Figs. 1 and 2a) distributed in three distinct portions with different apparent molecular weights and inhibited by different allosteric ligands (Fig. 2b). These isoenzymes were named DAHP synthases (Phe), (Tyr) and (Trp).

Standardization of elution patterns

In the course of the biochemical–genetic investigation of DAHP synthase[6] it was found that the elution pattern of isoenzymes (and therefore the apparent molecular weights) was constant using preparations from the same batch of mycelium but otherwise varied for the same strain. Variation was eliminated by removing unwanted small molecules before the sieving of protein began. This was done[6] by including a Biogel-P2 column (exclusion limit mol. wt. 1600) prior to the agarose column and resulted in DAHP synthase of higher apparent molecular weight. In contrast, equilibration of the P2–agarose duplex with phenylalanine, tyrosine and tryptophan (each 10 μM, phosphoenolpyruvate present at 0.1 mM) resulted in isoenzymes of

lower apparent molecular weight. The relationship between the various isoenzymes and the allosteric ligand environment is now examined for all possible combinations.

Whereas the data of Figs. 1 and 2 were obtained in phosphate buffer (pH 6.4), later experiments were done at pH 7.4 in the interests of greatest stability (P. HOFFMANN, personal communication) and to minimize "stimulations" and "cross-reactions" frequently found at pH 6.4 (ref. 6). The data presented is restricted to a sample illustrating the major affects, conclusions take into account the total laboratory experience.

Experiments in Tris–maleate

Experiments in Tris–maleate buffer were initiated since phosphate is a reaction product of DAHP synthase and to eliminate the tendency for the cofactor Co^{2+} (refs. 1, 4) to precipitate by reaction with phosphate buffers. In Tris–maleate there are considerable changes in the properties of fractions between first collection and storage and re-examination after freeze–thawing. In general, freeze–thawing activated many fractions particularly with respect to DAHP synthase (Phe) and sometimes altered inhibition patterns and created large stimulations by previous allosteric inhibitors. These changes, presumably due to interactions between protein subunits,

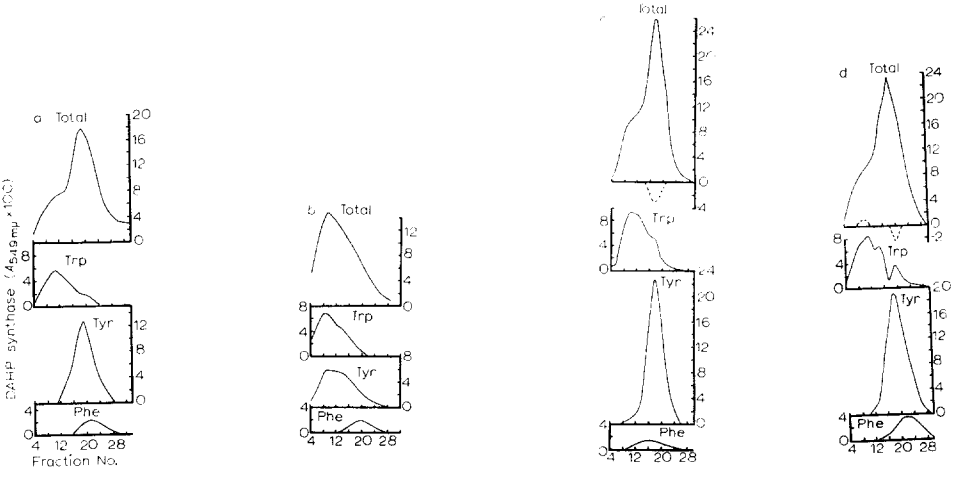


Fig. 3. Molecular sieving of *N. crassa* wild-type 74A DAHP synthase. All extracts prepared and eluted with 0.05 M Tris–maleate buffer (pH 7.4) containing 0.1 mM phosphoenolpyruvate and NaN_3 (0.02%, as sterilizing agent) *plus* allosteric ligands (each at 10 μM) as indicated. The latter were diluted to 1 μM in subsequent analysis of fractions so giving negligible inhibition unless further added for the determination of DAHP synthases (Phe), (Tyr) and (Trp) (see MATERIALS AND METHODS). All fractions were analyzed for total activity and the portions inhibited by phenylalanine, tyrosine and tryptophan individually. For the sake of clarity the actual experimental points are omitted but the activity profiles are drawn with the same attention to scatter as in Figs. 1, 2, 5 and 6. Total activity and the inhibited activities are drawn separately. In the total activity presentation a broken line is used to indicate when the sum of inhibited activities is less than the measured total activity (giving non-inhibited activity) or alternatively, exceeds the total activity (giving a negative activity regarded as evidence of stimulation or cross-inhibition by allosteric ligands). The protein front has been standardized as fraction zero. *a. Plus* phenylalanine, tyrosine and tryptophan. b. *Plus* phenylalanine and tryptophan. c. *Plus* tyrosine and tryptophan. Note indication that tryptophan partly inhibits DAHP synthase (Tyr). d. *Plus* phenylalanine and tyrosine. Note slight non-inhibited activity and indication that tryptophan partly inhibits at the overlap of DAHP synthases (Tyr) and (Phe).

varied greatly, accordingly, only reproducible data obtained without prior freezing is presented. This has the advantage that the sum of isoenzymic activities approximates the total activity measured without inhibitors (a condition not found for phosphate at this pH, see later) but the disadvantage that data for DAHP synthase (Phe) is quantitatively and qualitatively poor (Fig. 3). Good data for this isoenzyme was obtained in phosphate buffer which, however, provides additional problems (see later).

Without allosteric ligands the profile of total activity (not presented) resembled that of Fig. 4a with the activity predominantly at high apparent molecular weight. When all three allosteric ligands were present during sieving much of the activity moved to lower apparent molecular weight (Fig. 3a). Differential analysis showed that both DAHP synthases (Tyr) and (Phe) were in lower apparent molecular weight forms with profiles resembling those of standard proteins (see MATERIALS AND METHODS and ref. 6). In contrast, DAHP synthase (Trp) remained predominantly at high apparent molecular weight. Eluted with phenylalanine *plus* tryptophan, only DAHP synthase (Phe) moved to lower apparent molecular weight (Fig. 3b), whereas elution with tyrosine *plus* tryptophan moved DAHP synthase (Tyr) to lower apparent molecular weight (Fig. 3c). Although DAHP synthase (Phe) was barely detectable it was not in the lower apparent molecular weight form. On elution with phenylalanine *plus* tyrosine both DAHP synthases (Phe) and (Tyr) moved to lower apparent molecular weight leaving DAHP synthase (Trp) in the higher molecular weight region (Fig. 3d). The shape of the small tryptophan-inhibited region between DAHP synthases (Tyr) and (Phe) is inconsistent with protein sieving and is presumably cross-inhibition on the other isoenzymes (see the broken line in the total activity profile).

The results show that the presence of tyrosine converts DAHP synthase (Tyr) into a low apparent molecular weight form regardless of the presence of other allosteric ligands. A similar conclusion applies to phenylalanine and DAHP synthase (Phe) but none of the conditions changes DAHP synthase (Trp) to a lower apparent molecular weight.

Experiments in phosphate buffer

A series of experiments in phosphate buffer provides a better basis than the above for conclusions concerning DAHP synthase (Phe) but also provides new interpretive difficulties. In particular it was impossible to get good data for DAHP synthase (Trp) and in the Fig. 4 this part of the data is given for completeness only; secondly, the sum of the inhibited activities often falls far short of the total. It will be established that this non-inhibited activity is associated with the changes in apparent molecular weight of DAHP synthases (Phe) and (Tyr) directed by the allosteric ligands and should not be regarded as an independent fourth class of isoenzymes.

Elution in the absence of allosteric ligands resulted in DAHP synthases (Phe) and (Tyr) sieving predominantly in high apparent molecular weight forms (Fig. 4a). Considerable non-inhibited activity was associated with this region and its profile shows indications of being a mixture (Fig. 4a) as expected if different components were associated with DAHP synthases (Phe) and (Tyr) of differing apparent molecular weight.

Assuming a relationship between non-inhibited and inhibited activities, then the distribution of the proportion of activity between them influences the shape of the various profiles (see Fig. 4). Under these conditions, the total activity profile is

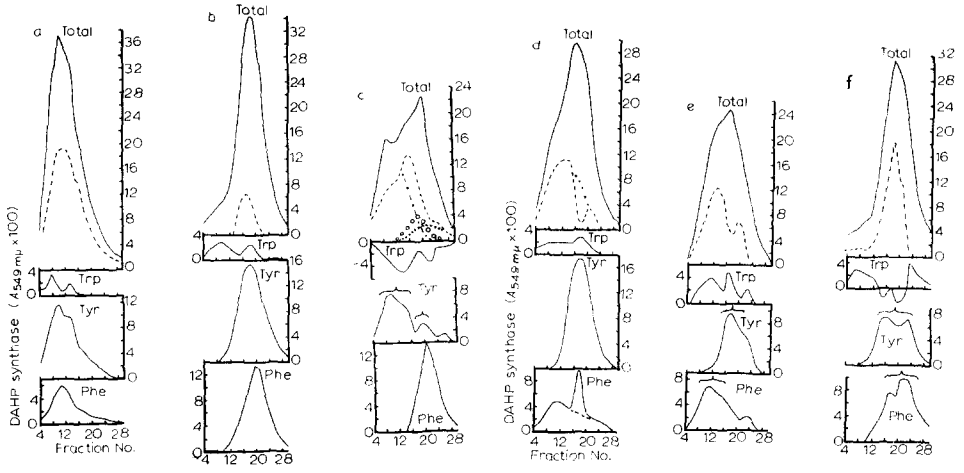


Fig. 4. Molecular sieving of *N. crassa* wild-type 74A DAHP synthase. All extracts prepared and eluted with 0.05 M KH_2PO_4–NaOH buffer (pH 7.4) containing 0.1 mM phosphoenolpyruvate and NaN_3 (0.02%) *plus* allosteric ligands (each 10 μM) as indicated. For other comments on general presentation see Fig. 3. Data for DAHP synthase (Trp) is unreliable and presented only for completeness (see text). Because the same isoenzyme consists of inhibited and non-inhibited contributions the separate profiles are mutually distorted. Total activity is not distorted and a careful comparison of this distribution with the non-inhibited and phenylalanine- and tyrosine-inhibited portions is necessary for a full understanding of the data. Brackets indicate positions of isoenzymes taking into account inhibited *plus* non-inhibited contributions. a. No allosteric ligands. b. *Plus* phenylalanine, tyrosine and tryptophan (each 10 μM). c. *Plus* phenylalanine. An attempt is made to interpret non-inhibited activity in terms of the high and low apparent molecular weight inhibited isoenzymes. — — —, total non-inhibited activity; ●—●, the postulated boundary associated with DAHP synthase (Tyr_{high}); ○ ○ ○, similarly for DAHP synthase (Tyr_{low}); ● ● ●, similarly for DAHP synthase (Phe_{low}). d. *Plus* tyrosine. The activity above the broken line in the DAHP synthase (Phe) plot is considered to be due to inhibition of DAHP synthase (Tyr) by phenylalanine; ● ● ●, represents the boundary when non-inhibited activity is corrected for this cross-inhibition. e. *Plus* tyrosine and tryptophan. f. *Plus* phenylalanine and tyrosine.

an accurate indication of shifts in apparent molecular weight, the inhibited activities indicate what portion has shifted but will vary in profile distortion in conjunction with the non-inhibited contribution.

Sieved in the presence of all three allosteric ligands, DAHP synthases (Tyr) and (Phe) moved to low apparent molecular weight (Fig. 4b). In this example the small amount of non-inhibited activity is associated mainly with DAHP synthase (Tyr) and allows the inhibited profiles to appear well formed.

On elution with phenylalanine, DAHP synthase (Phe) is at low apparent molecular weight (Fig. 4c). As always occurs in the absence of tyrosine, DAHP synthase (Tyr) is predominantly at high apparent molecular weight but does extend to the low molecular weight region indicating a mixture of forms. Non-inhibited activity is distributed broadly as expected and an attempt is made in Fig. 4c to rationalize the distribution. The sharp profile of DAHP synthase (Phe) and the distorted DAHP synthase (Tyr) profile indicates that non-inhibited activity is associated predominantly with the latter.

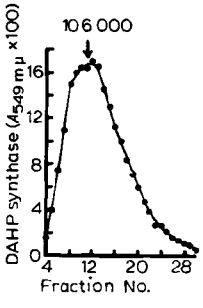
Elution with tyrosine converted DAHP synthase (Tyr) to low apparent molecular weight while DAHP synthase (Phe) remained predominantly at high apparent

molecular weight (Fig. 4d). In this example the DAHP synthase (Phe) profile is distorted by two factors: (a) a large contribution of non-inhibited activity at high molecular weight and (b) a sharp peak centered at approximately Fraction 18. This latter cannot represent a normal protein distribution and consequently is considered to be the result of cross-inhibition of phenylalanine on DAHP synthase (Tyr). This interpretation is consistent with the distribution of non-inhibited activity.

Elution with tyrosine *plus* tryptophan shifts DAHP synthase (Tyr) to low apparent molecular weight with the anticipated distribution of non-inhibited activity (Fig. 4e) and distortion of the inhibited profiles. A mixture of tyrosine *plus* phenylalanine shifted both isoenzymes to low apparent molecular weight and the distribution of non-inhibited activity follows as predicted (Fig. 4f). The large contribution of non-inhibited activity and the distorted profiles of inhibited activities are interrelated.

Correlation between apparent molecular weight and allosteric inhibition

The consensus of all experiments is that DAHP synthase (Tyr) can exist in high and low apparent molecular weight forms with peak activities in fractions corresponding to 106 000 and 68 000 apparent molecular weight. Unless phosphoenolpyruvate was included in the elution mixture both forms were unstable. Tyrosine at one tenth the concentration of phosphoenolpyruvate converts DAHP synthase (Tyr) to the lower form independently of other allosteric ligands. A similar conclusion applies to DAHP synthase (Phe) and phenylalanine, with apparent molecular weights of 99 000 and 57 000. In contrast, DAHP synthase (Trp) is relatively unaffected by the allosteric environment, is distributed with an average apparent molecular weight



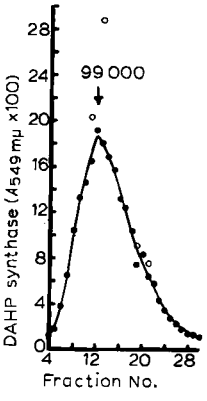


Fig. 5. Molecular sieving of allosteric inhibition (Tyr)-negative mutant *arom-6*$^{r(DH21)}$ *arom-7 arom-8*. The parent of the r(DH21) mutation was *arom-6*$^{+}$ *arom-7 arom-8*, that is, the only remaining DAHP synthase was DAHP synthase (Tyr). Elution system, phosphate buffer, phosphoenolpyruvate, NaN_3 *plus* tyrosine as for Fig. 4d but this totally non-inhibited enzyme remains predominantly in the high molecular weight form. Like DAHP synthase (Tyr) isoenzymes the peak tends to be flat-topped. Apparent molecular weight is 106 000 at maximum activity.

Fig. 6. Molecular sieving of allosteric inhibition (Phe)-negative mutant *arom-6 arom-7*$^{r(DH25)}$ *arom-8*. The parent of the r(DH25) mutation was *arom-6 arom-7*$^{+}$ *arom-8*, that is the remaining DAHP synthase was DAHP synthase (Phe). Elution system, phosphate buffer, phosphoenolpyruvate, NaN_3 *plus* phenylalanine as for Fig. 4c but this totally non-inhibited (but phenylalanine stimulated (○)) enzyme remains predominantly in the high molecular weight form. Apparent molecular weight is 99 000 at maximum activity.

greater than the others and is not dependent on phosphoenolpyruvate for detection.

The existence of allosteric inhibition-negative mutants[6] (strains insensitive to allosteric inhibition) allowed the relationship between apparent molecular weight and allosteric inhibition to be tested.

Extracts of phenylalanine- and tyrosine-resistant mutants were sieved in the presence of the now non-inhibiting ligand. It was found that conversion to the low molecular weight form did not occur (Figs. 5 and 6). The shape of the distributions indicates that there are low apparent molecular weight components but no more than is usual for wild-type in the absence of added tyrosine or phenylalanine (Fig. 4a). Estimated apparent molecular weights at maximum activity are 106 000 for the enzyme derived from DAHP synthase (Tyr) (Fig. 5) and 99 000 for that derived from DAHP synthase (Phe) (Fig. 6). These are in good agreement with the estimates for the high apparent molecular weight forms of DAHP synthases (Tyr) and (Phe) obtained from wild-type sieving patterns.

A particularly interesting result was obtained with *arom-7*$^{r(DH25)}$ (Fig. 6), the mutant no longer inhibited by phenylalanine. In all assay mixtures to which phenylalanine was added there was a greater total activity than in its absence. This is most clearly shown in samples taken from fractions at the peak of activity (Fig. 6). Thus, phenylalanine still binds with the altered enzyme although unable to cause a transition to low molecular weight. A similar but less marked effect was observed for the tyrosine-resistant mutant and this has also been observed in other work (see HALSALL AND DOY[6]).

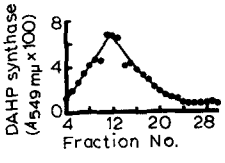


Fig. 7. Molecular sieving of allosteric inhibition (Trp)-negative mutant *arom-6 arom-7 arom-8*$^{r(DH19)}$. The parent of the r(DH19) mutation was *arom-6 arom-7 arom-8*$^{+}$, that is the remaining DAHP synthase was DAHP synthase (Trp). Elution system phosphate buffer, phosphoenolpyruvate, NaN_3 as Fig. 4 *plus* tryptophan (10 μM).

A tryptophan-resistant mutant was also examined. As shown by the analytical points in Fig. 7 the shape of the activity distribution is probably complex but as drawn the apparent molecular weight at maximum activity is 102 000. If components are present their likely apparent molecular weights are 130 000 and 77 000. These values are similar to estimates made from the wild-type enzyme.

DISCUSSION

The foregoing analysis of DAHP synthase by molecular sieving depends not only on size (molecular weight) but also shape and the possibility of interactions between isoenzymes (or with other proteins) during sieving, together with less obvious factors. Inspection of the data indicates a strong possibility that none of the major peaks represents a single molecular species. Accordingly the term apparent molecular

weight is emphasized and the numerical values are given to illustrate the extent of molecular changes rather than to assign molecular weight.

These reservations are important when considering the relationship between the higher and lower apparent molecular weight molecules. The ratio between the observed molecular weights at maximum activity for DAHP synthase (Tyr) (106 000 and 66 000) is 1.6 to 1 and for DAHP synthase (Phe) (99 000 and 56 000) is 1.8 to 1. In each example the lower apparent molecular weight form is regarded as representing a dissociation to a half molecule with the observed ratio modified by the factors discussed. In particular, it would seem very likely that the different forms could differ markedly in conformation.

As shown by the difference between the wild-type and allosteric-inhibition negative mutant enzymes (since found with many alleles, D. M. HALSALL, personal communication) dissociation is part of the normal mechanism of allosteric inhibition. It may be that the dissociated molecules are inactive but regain activity in the standard reaction mixtures. No assumption can be made regarding the molecular weight of the active enzyme.

Since phosphoenolpyruvate is required for stability of all forms of DAHP synthases (Phe) and (Tyr) and the allosteric ligands still react with the allosteric inhibition-negative mutant forms of enzyme, it is deduced that ligand binding is non-exclusive. This implies that it is the changes that follow ligand binding that are important for activity and activation on the one hand and inhibition and deactivation on the other.

The non-inhibited activity found in phosphate buffer presumably reflects the influence of the reaction product (phosphate) on the form of the enzyme under reaction conditions with added allosteric ligand. Either the dissociation cannot be sustained at 37° or else reactions that normally follow dissociation are interfered with. For convenience the assays were done at pH 7.4 as used for sieving, and there are indications that non-inhibition is less marked at pH 6.4. There is no reason to consider non-inhibited activity as a separately coded isoenzymic form since the triple mutant *arom-6 arom-7 arom-8* eliminates all DAHP synthase activity[6].

Non-inhibited activity is not confined to *N. crassa* preparations but has previously been concealed by the method of data presentation. Thus in the biochemical–genetic analysis of the *Escherichia coli* K12 enzyme WALLACE AND PITTARD[8,9] were concerned with the gross relationship between genes and the classes of inhibited isoenzymes rather than biochemical details. Hence their elution patterns from DEAE-cellulose are plotted as total activities only, with various portions labeled as tyrosine or phenylalanine inhibited. From other data in the paper these areas contain 20–32% of non-inhibited activity and the actual distribution of inhibited activities does not necessary follow the total activity. This non-inhibited activity should not be confused with the non-inhibited isoenzyme[9,10] thought to be derived from tryptophan-inhibited activity[11] *in vivo*.

As has been clear throughout our investigations of *Neurospora crassa* DAHP synthase there is a strong similarity between DAHP synthases (Phe) and (Tyr) but DAHP synthase (Trp) has rather different properties. In this study the most obvious differences are the latter's relative stability and the absence of gross changes in apparent molecular weight. Inspection of the profiles shows that the distribution of DAHP synthase (Trp) is complex with the probability of multiple conformers or other

forms. The investigation did not concentrate on this aspect of the system. In general the data has been dissected to emphasize that which resolves into a relatively simple picture. It is quite clear that in detail we are only just beginning to understand the intricities of DAHP synthase. Most remaining questions can only be solved by the use of highly purified forms of the enzyme or by careful comparisons between wild-type and mutant enzymes.

A detailed comparison with the *E. coli* enzyme would require more space than available. However, it should be pointed out that at present the *N. crassa* enzyme is unique in several ways. Firstly, it is easy to find a tryptophan-inhibited isoenzyme, secondly, there is evidence for cooperation between ligands and thirdly, the dissociations of DAHP synthases (Phe) and (Tyr). While this paper was under revision to reduce size it was noticed that STAUB AND DÉNES[12] state that the molecular weight of *E. coli* K12 DAHP synthase (Phe) (160 000 but by sieving, purification 160-fold, no criteria for purity given) is unaltered by phosphoenolpyruvate or by phenylalanine. Whereas the *E. coli* enzyme was originally described as without a heavy-metal requirement, the Hungarian workers[12,13] have now established a Co^{2+} requirement as reported for *N. crassa*[1,4]. It remains an open question as to whether the *N. crassa* enzyme has evolved differently since uniqueness may lie in the difference of experimental approach. In this laboratory we have preferred to first explore the crude enzyme for an overall assessment and to refrain from estimation of kinetic constants *etc.*, until a very high degree of purity of individual isoenzymes is obtained. At present DAHP synthase (Tyr) is at least 6000-fold purified (P. HOFFMANN, personal communication) and it is hoped that meaningful kinetic measurements may be possible with the limited analytical procedure available for this enzyme.

A pointer that *N. crassa* may differ from *E. coli* lies in the greater degree of biofunctional organization or gene interaction detected in *N. crassa*. For example, there is a multi (5) functional protein (aggregate) of common path *N. crassa* enzymes[14] but not in *E. coli*[15]. Recent work (C. H. DOY, unpublished) shows that properties of *N. crassa* DAHP synthase are changed by mutations previously thought to affect only the common path aggregate and chorismate synthase. This observation unifies the entire common path sequence of enzymic events.

Investigation of the nutritional role of the common path intermediate shikimate reveals other pleiotropic gene affects (C. H. DOY, unpublished). Of the six genes concerned with pre-shikimate reactions only *arom-1* alleles are able to utilize shikimate in place of the full aromatic end product requirement. The positive growth response of *arom-1* alleles is reversed by introducing the *qa-1* gene. This codes for three of the inducible quinic acid degradative pathway enzymes including a dehydroquinase able to mimic the biosynthetic constitutive dehydroquinase[16]. These complex gene interactions suggest a degree of interaction between polypeptides *in vivo* (biofunctional organization) that has escaped detection by analysis based on experiments *in vitro* with the "purified" components of aromatic biosynthesis.

It is anticipated that many of the peculiarities of DAHP synthase will eventually be attributed to inter-isoenzymic and intra-pathway functional organization that can only be properly understood by attention to the organism as a living entity as well as a source of interesting separable proteins.

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