Differential Repression of Arylsulphatase Synthesis in *Aspergillus oryzae*

By GARY R. J. BURNS and COLIN H. WYNN

Department of Biochemistry, University of Manchester, Manchester M13 9PL, U.K.

(Received 28 February 1977)

1. The activities of the three arylsulphatases (arylsulphate sulphohydrolase, EC 3.1.6.1) of *Aspergillus oryzae* produced under a variety of repressing and non-repressing conditions were determined. 2. These enzymes exhibit different sensitivities to repression by inorganic sulphate. 3. Arylsulphatase I, but not arylsulphatases II and III, exhibits a transient de-repression in the early growth phase in sulphate media. 4. When the fungus is cultured in repressing media and subsequently transferred to non-repressing media, the synthesis of the three enzymes is non-co-ordinate. 5. Growth of the fungus in media containing choline *O*-sulphate or tyrosine *O*-sulphate as the sole source of sulphur results in complete de-repression of arylsulphatase I, but the synthesis of arylsulphatases II and III is essentially fully repressed. 6. The marked similarities between the repression characteristics of arylsulphatases II and III, contrasted with those of arylsulphatase I, indicate that the genetic locus of arylsulphatase I is distinct from that of arylsulphatases II and III, suggesting that there are distinct physiological roles for the enzyme.

Despite the number of studies of the regulation of arylsulphatase (arylsulphate sulphohydrolase, EC 3.1.6.1) synthesis in micro-organisms, little consideration has been given to possible differences in such control mechanisms among the multiple arylsulphatases of any given organism.

The multiplicity of arylsulphatases in micro-organisms was first demonstrated by Cherayil & van Kley (1962, 1963), and evidence was obtained which indicated that these might be subject to different regulatory controls. Unfortunately little detail has been published about these differences, but Cherayil & van Kley (1962, 1963) did report that under their conditions of growth, *Aspergillus oryzae* normally produced two arylsulphatases, the proportions of which varied with the amount of water added to the cultures, which were grown on moist wheat bran. A small quantity of a third enzyme was present, but the proportions of this could be increased by growth in the presence of molybdate or tungstate ions, which are both competitive inhibitors of the enzymes.

Harada & Spencer (1962) made a detailed study of the effect of a wide variety of sulphur compounds on arylsulphatase synthesis by a number of fungi, and general repression characteristics were established. Unfortunately, only total arylsulphatase activity was determined.

Harada (1964) studied arylsulphatase synthesis in *Pseudomonas aeruginosa*, and although total arylsulphatase synthesis was measured, it was observed that hydrolysis of tyramine *O*-sulphate and tyrosine *O*-sulphate by cell-free extracts only occurred where one of these esters had served as the sulphur source, despite extensive hydrolysis of *p*-nitrophenyl sulphate

when other non-repressing sulphur compounds were utilized for growth. These observations indicated that this organism may produce an inducible as well as a repressible arylsulphatase.

Previous studies have shown that the three arylsulphatases of *A. oryzae* exhibit markedly distinct kinetic properties and substrate specificities. Thus phenolphthalein disulphate and tyrosine *O*-sulphate are substrates only for arylsulphatase I (Burns & Wynn, 1975, 1977), whereas arylsulphatase II exhibits an unusual phenol sulphotransferase activity (Burns & Wynn, 1975).

These striking differences, together with the above observations on control of arylsulphatase synthesis, suggested that the three enzymes might be subject to distinct regulatory mechanisms.

We have developed a rapid assay method based on the distinct kinetic properties of the enzymes, which allows the separate determination of each enzyme present in crude cell extracts (Burns & Wynn, 1977). In the present paper we report the application of this assay to the measurement of the three enzymes produced under a variety of cultural conditions.

Materials and Methods

Culture conditions

Spores were obtained from slopes of *A. oryzae* grown as described previously (Burns & Wynn, 1975). Inocula were prepared by flooding slopes with a sterile solution of Tween-20 (0.025%, w/v) and filtering the resulting suspension through a double layer of sterilized muslin. The spore concentration was adjusted to approx. 10^7 /ml.

The sulphur-free minimal medium used for growth in submerged culture has been described previously (Burns & Wynn, 1975). This medium was supplemented as necessary with a variety of sulphur compounds. Where these were labile to autoclaving, solutions were sterilized by filtration through membrane filters (0.45 μm pore diameter; Oxoid Ltd., Basingstoke, Hants. RC24 0PW, U.K.).

Spore suspension (10 ml) was inoculated into 150 ml of the appropriate medium in duplicate 500 ml conical flasks, and the culture was shaken at 180 rev./min and 28°C in an orbital shaker.

De-repression of arylsulphatase synthesis

Mycelium from repressed cultures was collected by centrifugation at 2000g for 10 min in sterile centrifuge bottles. The supernatant was decanted and the mycelium was resuspended in the same volume of sterile sulphur-free medium. This procedure was repeated twice and the mycelium finally resuspended in sulphur-free medium. Portions (200 ml) were dispensed into a number of 500 ml conical flasks and cultured as above. In some experiments, cycloheximide (100 $\mu\text{g}/\text{ml}$) was included in the culture medium.

Preparation of homogenates and arylsulphatase assay

Cultures were filtered at the pump and the mycelial pads were washed twice by resuspension and filtration with 200 ml of ice-cold water. Acetone-dried powders were prepared as described previously (Burns & Wynn, 1975).

The acetone-dried powders were resuspended in ice-cold buffer (10 mg/ml; 0.2 M-Tris adjusted to pH 6.9 with 0.2 M-maleic acid) and homogenized in a glass tube with a mechanically driven Teflon pestle. After overnight dialysis at 4°C against 100 vol. of the above buffer, the homogenates were assayed for each enzyme by using a differential assay procedure (Burns & Wynn, 1977). Before the absorptions of the solutions were read, insoluble material was removed by centrifugation at 2000g for 10 min and filtration through Millipore membrane filters (0.45 μm pore diameter).

Chemicals

The potassium salt of tyrosine *O*-sulphate was prepared by the method of Dodgson *et al.* (1959). Choline *O*-sulphate was prepared by the method of Bellenger *et al.* (1968). L-Methionine, taurine (2-aminoethanesulphonic acid) and cycloheximide were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Tween-20 was obtained from Koch-Light Laboratories, Colnbrook SL3 0BZ, Bucks., U.K.

Results

Arylsulphatase synthesis during germination and growth in repressing media

Several enzymes of sulphur metabolism in fungi have been shown to exhibit a developmental regulation, with their activities varying according to the developmental stage of the organism. Examples include sulphate permease (Marzluf, 1970) and choline sulphatase (McGuire & Marzluf, 1974a). Growth curves and arylsulphatase activities during culture in repressing media are shown in Fig. 1. For clarity, the data are expressed as the percentage of the maximum specific activity observed in cultures grown in sulphur-free medium. Defining one enzyme unit as that amount of enzyme producing an increase in A_{402} of 1 unit/h under the assay conditions, the following maximum specific activities were observed for each enzyme: arylsulphatase I, 1.0 unit/mg of powder; arylsulphatase II, 44 units/mg of powder; and arylsulphatase III, 2.8 units/mg of powder.

The most striking feature of the data is a transient decrease in repression of arylsulphatase I during the exponential growth phase in sulphate medium, the activity of this enzyme reaching approx. 70% of the maximally de-repressed value. No similar de-repres-

Fig. 1. *Synthesis of arylsulphatase during germination and growth in repressing media*

Cultures were grown in medium supplemented with either 1 mM- K_2SO_4 (open symbols) or 1 mM-L-methionine (solid symbols) and harvested at intervals of time after inoculation with the spore suspension. Acetone-dried powders were prepared and assayed for arylsulphatase I ($\square$, $\blacksquare$), arylsulphatase II ($\circ$, $\bullet$) and arylsulphatase III (Δ , $\blacktriangle$). ($\bigcirc$) Growth, expressed as dry weight.

Fig. 2. Variation in degree of repression with sulphate concentration

Cultures were grown in minimal medium supplemented with K_2SO_4 over the concentration range 0–1 mM. Mycelia were harvested after 65 h growth and acetone-dried powders were prepared and assayed for arylsulphatase I (■), arylsulphatase II (●) and arylsulphatase III (▲). (○), Growth, expressed as dry weight.

Fig. 3. De-repression of arylsulphatase synthesis

Cultures were grown for 65 h in minimal medium supplemented with 5 mM- K_2SO_4 and then transferred to sulphur-free medium. Mycelia were subsequently harvested after intervals of time and acetone-dried powders prepared and assayed for arylsulphatase I (■), arylsulphatase II (●) and arylsulphatase III (▲). (○), Growth, expressed as dry weight.

sion of arylsulphatases II and III can be observed. In the presence of methionine, similar patterns of enzyme synthesis were observed, although arylsulphatase I reaches only 20% of its maximally de-repressed value.

Effect of inorganic sulphate concentration on arylsulphatase synthesis

The data from the previous experiment indicated that the synthesis of the three enzymes was not co-ordinate. To investigate the possibility that the concentrations of the sulphur pools of *A. oryzae* might also exert distinct influences over the synthesis of the enzymes, experiments were carried out with cultures grown in the presence of various concentrations of inorganic sulphate. The results are illustrated in Fig. 2. Clearly there are considerable differences in the sensitivities of the three enzymes to repression by inorganic sulphate: arylsulphatase I shows a steady increase as the sulphate concentration is decreased, whereas arylsulphatase II does not increase appreciably until concentrations of sulphate are less than 0.5 mM. Arylsulphatase III activity varies in a manner more characteristic of arylsulphatase II than of arylsulphatase I, although it can be detected at higher concentrations of sulphate than can arylsulphatase II.

De-repression of arylsulphatase synthesis

The pronounced differences in sensitivity of the three enzymes to repression by inorganic sulphate indicated that non-co-ordinate synthesis of the enzymes might be expected on transfer of the fungus from repressing to non-repressing media, since the concentrations of the sulphur pools would decrease under such conditions. The results of these experiments are shown in Fig. 3.

The growth of fungus after transfer to de-repressing media exhibits a biphasic pattern, with substantial growth occurring during the latter, slower phase. The development of enzyme activity during de-repression is characterized by a rapid increase in arylsulphatase I synthesis during the initial growth phase. This enzyme was synthesized after a very short lag phase and reached its maximally de-repressed activity before the end of the initial growth period.

The synthesis of arylsulphatases II and III was characterized by a lag phase of 2–4 h. After nearly 50 h the activities of these enzymes had still reached only 20–30% of the maximum specific activities observed when cultures were grown from spores in sulphur-free medium.

When cycloheximide was included in the medium, the specific activities of the three enzymes did not increase.

Table 1. *Specific repression of arylsulphatases II and III by choline O-sulphate and tyrosine O-sulphate*
 Values in parentheses indicate the specific activities of the enzymes expressed as a percentage of the maximum activity obtained during growth in the absence of any added sulphur compounds.

Sulphur source	Dry wt. (g)	Arylsulphatase specific activity (units/mg)		
		I	II	III
K ₂ SO ₄	1.28	0.06 (6)	0.12 (0.3)	0.04 (1)
Choline O-sulphate	1.29	0.86 (86)	0.22 (0.5)	0.20 (7)
Tyrosine O-sulphate	1.23	0.87 (87)	0.76 (2)	0.42 (15)
Taurine	1.25	1.00 (100)	13.40 (30)	1.60 (57)
Choline O-sulphate+K ₂ SO ₄	1.26	0.05 (5)	0.10 (0.2)	0.06 (1.5)
Tyrosine O-sulphate+K ₂ SO ₄	1.28	0.06 (6)	0.12 (0.3)	0.02 (0.5)
Taurine+K ₂ SO ₄	1.27	0.04 (4)	0.11 (0.3)	0.06 (1.5)

Specific repression of arylsulphatases II and III by choline O-sulphate and tyrosine O-sulphate

The results of the previous experiment suggested that different rates of depletion of components of the sulphur pool were responsible for the non-co-ordinate de-repression of arylsulphatase synthesis.

Choline O-sulphate is an important storage form of sulphur in many fungi (Spencer & Harada, 1960; Takebe, 1960). This compound was utilized in the present studies, since under de-repressing conditions its utilization is critical in maintaining adequate concentrations of reduced sulphur for growth. For comparative purposes, cultures were also examined for arylsulphatase synthesis after growth in media containing sulphate, a repressor of many enzymes of sulphur metabolism, taurine, a non-repressing sulphur source, and tyrosine O-sulphate, a potential substrate for the enzymes.

Cultures were grown in minimal media supplemented with the various sulphur sources at a concentration of 1 mM. After 65 h, acetone-dried powders were prepared and assayed as described in the Materials and Methods section. The results of these studies are shown in Table 1. The dry weights of the mycelia were similar, regardless of the sulphur source used, indicating that all could serve as the sole source of sulphur. As noted above, full repression of all three enzymes was produced by growth in K₂SO₄-supplemented medium. When taurine was the sole sulphur source, arylsulphatase I was fully de-repressed, and a marked but not complete de-repression of arylsulphatases II and III could be observed.

Growth in the presence of choline O-sulphate or tyrosine O-sulphate produced a quite different pattern of arylsulphatase synthesis. Arylsulphatase I synthesis was completely de-repressed during growth in the presence of both sulphate esters, but essentially full repression of arylsulphatase II and III was observed. When inorganic sulphate was included with choline O-sulphate or tyrosine O-sulphate, the pattern of arylsulphatase activity was identical with that observed with inorganic sulphate only, indicating

that the synthesis of arylsulphatase I in the presence of the sulphate esters was not the result of a specific induction by these compounds. Assay of homogenates prepared from a mixture of acetone-dried powders from cultures grown with tyrosine O-sulphate with and without inorganic sulphate produced additive results, indicating that the observed activities were due to effects on the synthesis of the enzymes rather than inhibition by endogenous compounds.

Discussion

The results of the present study indicate that significant differences exist in the control mechanisms regulating the synthesis of the three arylsulphatases of *A. oryzae*, within the overall context of repression by a variety of sulphur compounds such as inorganic sulphate, cysteine and methionine.

Apart from differences in sensitivity to repression by inorganic sulphate, some other distinctions can be made. During germination and outgrowth of mycelia in repressing conditions the activities of the three enzymes vary. The most notable feature of this variation is a transient increase in arylsulphatase I synthesis during the exponential growth phase in sulphate medium. When methionine was used as the sole source of sulphur, this early increase in arylsulphatase synthesis was less pronounced. No de-repression of synthesis of arylsulphatase II and III was observed during growth in either medium.

The behaviour of arylsulphatase I in this respect is very similar to that of choline sulphatase in *Neurospora crassa*. In this organism a rapid and early synthesis of choline sulphatase is observed during conidial germination under de-repressing conditions, and moreover, the enzyme was also synthesized for a brief period in germinating conidia, even in the presence of repressing concentrations of inorganic sulphate (McGuire & Marzluf, 1974a).

It seems probable that the transient increase in arylsulphatase synthesis during the exponential

growth phase of *A. oryzae* reflects the general activation of metabolism that is associated with the breaking of dormancy. Nevertheless, the restriction of this synthesis to arylsulphatase I again indicates a difference in the control mechanisms regulating the synthesis of the three enzymes, which may be related to differences in the complex sulphur pools which in *N. crassa* have been shown to exhibit developmental regulation (McGuire & Marzluf, 1974b).

During de-repression after transfer from repressing to non-repressing media, the synthesis of arylsulphatase I proceeds rapidly to its maximum value, whereas synthesis of arylsulphatases II and III proceeds only after an initial lag phase of several hours and did not achieve the maximum possible values after 50 h further growth.

The ability of many fungi to store sulphur as choline *O*-sulphate has been noted above. It is clear that growth in sulphur-free medium will result in rapid depletion of readily assimilable forms of sulphur. Such depletion would result in the degradation of storage forms and thus the attainment of lower concentrations of potential repressors such as cysteine and methionine. The development of arylsulphatase I activity during de-repression can thus be interpreted in terms of the rapid depletion in concentrations of readily assimilated sulphur compounds. The concentration of repressors, however, can be maintained initially at a value high enough to result in the repression of arylsulphatases II and III, but as growth continues, storage forms become depleted and end products of sulphur metabolism gradually decrease below the concentration required to maintain full repression of arylsulphatases II and III.

When *A. oryzae* was grown with choline *O*-sulphate as the sole source of sulphur, the pattern of arylsulphatase synthesis was consistent with the foregoing in that arylsulphatase I was fully de-repressed while essentially full repression was maintained on arylsulphatases II and III. Choline *O*-sulphate did not generally repress arylsulphatase synthesis in a number of fungi (Harada & Spencer, 1962) or in *Pseudomonas C₁₂B* (Fitzgerald & Payne, 1972). Growth of *A. oryzae* with tyrosine *O*-sulphate as the sole source of sulphur produced similar patterns of arylsulphatase synthesis, and no evidence was obtained that this ester could induce any of the enzymes, as has been observed in *Pseudomonas aeruginosa* (Harada, 1964). It has been shown that tyrosine *O*-sulphate is hydrolysed only by arylsulphatase I (Burns & Wynn, 1975), and since extensive hydrolysis of the ester would result in repression of this enzyme the hydrolysis is to some extent self-regulating, and end-products of sulphur metabolism are unlikely to accumulate to concentrations much greater than that required to sustain growth. Thus a similar pattern of arylsulphatase synthesis is predicted for growth in media containing either ester.

The non-co-ordinate synthesis of the three arylsulphatases could be the result of distinct, non-linked genetic loci coding for arylsulphatase I and arylsulphatases II and III. Alternatively, it is possible that activation of the gene products is required for expression of arylsulphatases II and III, and that this activation is inhibited by some sulphur metabolite. Apte *et al.* (1974) have studied the kinetics of de-repression of the two arylsulphatases of *Aspergillus nidulans*. Their results indicate that the active enzyme is made in two steps, the first but not the second step requiring synthesis of protein *de novo*. Both these steps are separately controlled by sulphur metabolites. When the time-course of synthesis was studied, it was found that the two enzymes were not synthesized simultaneously. Whichever alternative is correct, the profound differences in control of arylsulphatase synthesis imply distinct physiological functions for the enzymes, and indicate that there may be distinct substrates.

Having regard to the demonstrations that the mammalian arylsulphatases A and B function *in vivo* as cerebroside sulphatase (Jerfy & Roy, 1973; Stins-hoff & Jatzkewitz, 1975) and chondrosulphatase (Matalon *et al.*, 1974; O'Brien *et al.*, 1974) respectively, the limited specificity of the microbial arylsulphatases so far demonstrated *in vitro* should be regarded with some caution.

The maintenance of repression of arylsulphatases II and III until the most extreme conditions of sulphur depletion are attained may indicate that their substrates are important components of the organism which can only be spared when no other sources are available to provide sulphur for maintenance of protein synthesis. It is noteworthy in this respect that Schmit & Brody (1976) have observed that the conidia from a mutant strain of *Neurospora crassa* which produces constitutive synthesis of arylsulphatase are osmotically fragile, suggesting that constitutive synthesis of the enzymes of sulphur metabolism during conidiation results in the formation of either a defective plasma membrane or an altered cell wall.

Further examination of the sulphur pools of *A. oryzae* and correlation of pool sizes with arylsulphatase activities, in conjunction with a widely spread search for potential substrates, should permit progress to be made in defining the function of these enzymes.

G. R. J. B. thanks the Medical Research Council for a Research Studentship. We also thank Dr. J. W. Fitzgerald of the Department of Microbiology, University of Georgia, Athens, GA, U.S.A., for critical revision of the manuscript.

References

- Apte, B. N., Bhavsar, P. N. & Siddiqi, O. (1974) *J. Mol. Biol.* **86**, 637–648

- Bellenger, N., Nissen, P., Wood, T. C. & Segel, I. H. (1968) *J. Bacteriol.* **96**, 1574-1585
- Burns, G. R. J. & Wynn, C. H. (1975) *Biochem. J.* **149**, 697-705
- Burns, G. R. J. & Wynn, C. H. (1977) *Biochem. J.* **166**, 415-420
- Cherayil, J. D. & van Kley, H. (1962) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **21**, 230
- Cherayil, J. D. & van Kley, H. (1963) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **22**, 241
- Dodgson, K. S., Rose, F. A. & Tudball, N. (1959) *Biochem. J.* **71**, 10-15
- Fitzgerald, J. W. & Payne, W. J. (1972) *Microbios* **6**, 147-156
- Harada, T. (1964) *Biochim. Biophys. Acta* **81**, 193-196
- Harada, T. & Spencer, B. (1962) *Biochem. J.* **82**, 148-156
- Jerfy, A. & Roy, A. B. (1973) *Biochim. Biophys. Acta* **293**, 178-190
- Marzluf, G. A. (1970) *Arch. Biochem. Biophys.* **138**, 254-263
- Matalon, R., Arbogast, B. & Dorfman, A. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1450-1457
- McGuire, W. H. & Marzluf, G. A. (1974a) *Arch. Biochem. Biophys.* **161**, 360-368
- McGuire, W. G. & Marzluf, G. A. (1974b) *Arch. Biochem. Biophys.* **161**, 570-580
- O'Brien, J. F., Cantz, M. & Spranger, J. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1170-1177
- Schmit, J. C. & Brody, S. (1976) *Bacteriol. Rev.* **40**, 1-41
- Spencer, B. & Harada, T. (1960) *Biochem. J.* **77**, 305-315
- Stinshoff, K. & Jatzkewitz, H. (1975) *Biochim. Biophys. Acta* **377**, 126-138
- Takebe, I. (1960) *J. Gen. Appl. Microbiol.* **6**, 83-89