N-Acetyl-6-hydroxytryptophan oxidase, a developmentally controlled phenol oxidase from *Aspergillus nidulans*

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We have purified a specific phenol oxidase which is produced during conidiophore development in the fungus *Aspergillus nidulans*. Two active forms (A and B) have molecular masses of 50 and 48 kDa respectively; they have identical N-termini (24 residues). We have analysed the metal ion content of the B form; it is unusual in consisting of one zinc and two copper atoms per molecule. A temperature-sensitive mutant (*ivoB192*) produces a thermolabile enzyme, implying that *ivoB* is the structural locus. The natural substrate of the enzyme is *N*-acetyl-6-hydroxytryptophan, but it can be assayed colorimetrically or polarographically using hydroquinone monomethyl ether (HME) as substrate. It will also oxidize *p*-cresol, but not tyrosine, 3,4-dihydroxyphenylalanine or *o*-methoxyphenol. Colour development with HME substrate is strongly enhanced by high ammonium ion concentrations. Activity against HME is inhibited by 2,3-dihydroxynaphthalene, phenylhydrazine, diethyl dithiocarbamate and 8-hydroxyquinolene.

Introduction

The upper conidiophore, vesicle, metulae and phialides of the asexual sporulation apparatus of the mould *Aspergillus nidulans* are normally pigmented grey-brown. This pigmentation requires the activity of two genes, *ivoA* and *ivoB*, which when mutated give 'ivory' (colourless) but otherwise normal conidiophores (Clutterbuck, 1969). *ivoA* is involved in the biosynthesis of a novel phenol oxidase substrate, *N*-acetyl-6-hydroxytryptophan (AHT; see Fig. 1a) (McCorkindale *et al.*, 1983) while *ivoB* codes for the conidiophore-specific oxidase (AHTase) which we here describe in detail.

Methods

Standard culture and genetical methods for *A. nidulans* are described by Pontecorvo *et al.* (1953) and Clutterbuck (1974). Strains were derivatives of those in the Glasgow collection.

Production and extraction of AHTase. The enzyme was obtained from *A. nidulans* surface cultures (Clutterbuck, 1972), consisting of 20 ml of

Aspergillus complete medium agar (Clutterbuck, 1974) overlaid with 5 ml of liquid complete medium containing an inoculum of 10^4 conidia per dish of strain AJC7.46, genotype *ivoA1*; *ornB7* *brlA42*. (*brlA42* halts conidiophore development at the stage at which AHTase production is maximal and *ivoA* doubles AHTase yield, probably because of the absence of tanning; Clutterbuck, 1990). The plates were incubated at 37 °C for 5 d, after which the mycelium was scraped off and shaken with 2 ml per plate of extraction buffer (20 mM-Tris, pH 7.4, containing 1 mM-phenylmethylsulphonyl fluoride protease inhibitor). The extract was filtered through a coarse filter and centrifuged at low speed to remove mycelium.

The temperature-sensitive mutant *ivoB192* was isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis of an *abaA6* strain (Clutterbuck, 1969). A recombinant substituting *brlA7* for *abaA6* as aconidial background was used for enzyme studies. Mycelium of this strain, along with the *brlA7* *ivoB*⁺ control, was grown for 1 d at 37 °C and for 2 d at 22 °C, and ground with sand in a cold mortar. The extract was centrifuged at 4 °C, and kept on ice.

Purification of AHTase. This was done at room temperature. All solutions were made up in extraction buffer. Ion-exchange chromatography was carried out using a 280 × 16 mm column of DEAE-Sephacrose Cl-6B, at a constant flow rate of 50 ml h⁻¹, eluting with a

Fig. 1. Substrates for AHTase. (a) *N*-Acetyl-6-hydroxytryptophan (AHT); (b) hydroquinone monomethyl ether (HME).

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Abbreviations: AHT, *N*-acetyl-6-hydroxytryptophan; AHTase, *N*-acetyl-6-hydroxytryptophan oxidase; HME, hydroquinone monomethyl ether.

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linear 0 to 0.5 M-NaCl gradient. The five fractions with highest AHTase specific activity from the ion-exchange column were pooled and made up to 4 M-NaCl before application to a 150 × 9 mm phenyl-agarose column previously equilibrated with extraction buffer. The column was washed with 30 ml of 4 M-NaCl (flow rate 10 ml h⁻¹) and AHTase activity was eluted with a linear 4 to 0 M-NaCl gradient.

FPLC was carried out using a Phenyl-Superose column HR 5/5 (Pharmacia) which was washed with extraction buffer until a constant basal A_{280} level was observed. The sample in 4 M-NaCl was loaded onto the column and washed with 4 M-NaCl. A linear 4 to 0 M-NaCl gradient was then applied to the column, and 0.5 ml fractions were collected and assayed for AHTase activity. Protein levels were assessed by measuring A_{280} on a Single Path Monitor UV-1 (Pharmacia). A constant 0.4 ml min⁻¹ flow rate was maintained for all operations. Samples were concentrated and desalted using Centricon centrifugal micro-concentrators.

Protein assay. This followed the method of Bradford (1976). Proteins on gels were stained with Coomassie blue. Silver staining of peptide gels used the method of Wray *et al.* (1981). Purification was monitored using SDS-PAGE in 9% (w/v) acrylamide gels (Laemmli, 1970). For non-denaturing SDS gels, samples were prepared without boiling and with no 2-mercaptoethanol in the sample buffer. Size markers for gels were obtained from Sigma: bovine serum albumin (66 kDa); egg albumin (45 kDa); rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa); bovine erythrocyte carbonic anhydrase (29 kDa); trypsinogen (24 kDa); soybean trypsin inhibitor (20 kDa); and bovine lactalbumin (14 kDa).

Metal analysis. This was performed by Dr D. J. Halls, Glasgow Royal Infirmary Institute of Biochemistry. Copper and chromium were detected by electrothermal atomic absorption spectrometry and zinc by flame atomic absorption spectrometry.

N-terminal sequencing. Samples of both forms, 250 pmol (11 µg) of the A-form and 500 pmol (22 µg) of the B-form, were washed three times with 0.1% trifluoroacetic acid, using Centricon filtration, and freeze-dried. The samples were sequenced by the SERC facility at the Department of Biochemistry, University of Aberdeen, UK.

Colorimetric AHTase assay. The assay mixture contained 67.2 mM-hydroquinone monomethyl ether (HME), 0.6 mM-catechol, 0.5 M-ammonium tartrate (pH 7.0). Increase in A_{470} was followed at 37 °C in a cell of 1 cm pathlength with a Pye Unicam spectrophotometer.

Ascorbate delay assay. Low concentrations of ascorbate, e.g. 0.1 mM, were added to the colorimetric assay and the additional time lag before the start of colour development (compared to ascorbate-free controls) was measured.

Polarographic assay. This was performed in an oxygen electrode cell supplied by Rank Bros, Bottisham, Cambridge, UK.

Preliminary substrate test. Potential substrates were initially tested for colour reaction with colonies of strains blocked at the conidiophore stage by means of the *brlA42* or *abaA1* mutations (Clutterbuck, 1969) and rendered colourless by an *ivoA* mutation, using a similar strain carrying the *ivoB63* mutation as control. Some substrates were further tested using the polarographic assay.

Thermolability test. Extract samples were transferred to 60 °C for the stated time and then back to ice before assaying colorimetrically with HME substrate at 25 °C.

Diazotized sulphanilic acid test for 6-hydroxyindoles. This was done as described by Jepson (1960) and Jepson *et al.* (1962). One ml of sulphanilic acid reagent (0.9 g sulphanilic acid in 9 ml concentrated hydrochloric acid, diluted 1/10 with water) was added to 0.1 ml 5% aqueous sodium nitrite, and left on ice for 5 min. A crystal of

ammonium sulphamate was added to remove excess nitrous acid, 0.1 ml of the mixture was diluted with 2.9 ml 1 M-HCl, and 0.1 ml of the extract to be tested was added. The pink colour was allowed to develop in the dark for 20 min, and read as A_{530} . Jepson *et al.* (1962) found that an A_{530} of 1.0 was equivalent to 13 µg of 6-hydroxyindole. To test colonies for 6-hydroxyindoles, they were overlaid with filter paper soaked in the reagent.

Results

Enzyme extraction, purification and composition

AHTase is an extracellular enzyme of unusual stability. Extraction and purification were carried out at room temperature, and extracts stored at 4 °C retained full activity for 3 weeks. Activity was also retained after electrophoresis through non-denaturing SDS-polyacrylamide gels.

A single extraction by shaking mycelium from surface cultures with buffer released approximately 45% of AHTase activity and 25% of total protein (as estimated from mycelium ground in a mortar). A high degree of purification leaving only one major contaminant (Fig. 2, lanes 3 and 4) was achieved in only two steps: Table 1 shows the results of purification of AHTase through ion-exchange and hydrophobic-interaction chromatography. The latter separated two peaks of activity (forms A and B) which were also distinguishable by SDS-PAGE (Fig. 2).

For further purification, active fractions from the phenyl-agarose column were concentrated, pooled and passed through an FPLC Phenyl-Superose column. Electroelution from an SDS-polyacrylamide gel was employed as a final step for the A form. We estimate from Fig. 2 that the sizes of the two forms are 50 kDa (A-form) and 48 kDa (B-form).

Peptide mapping and N-terminal sequence

The structural similarity between the two forms of AHTase was demonstrated by peptide mapping (Cleveland, 1983). Both A and B forms were digested with chymotrypsin. Peptides were separated on a 15% (w/v) SDS-polyacrylamide gel and detected with silver staining. Both forms yielded three fragments, of sizes 13, 15, and 19 kDa for the A form and 13, 15 and 17 kDa for the B form (results not shown).

Fig. 3 shows the N-terminal sequences of the B form. Of the first 34 residues, 31 were resolved unambiguously. For the A-form, residues 1 to 24 were resolved, with ambiguities at positions 5, 8, 9, 16 and 22; the resolved residues however, were identical with those shown for the B-form.

Fig. 2. Purification of AHTase. Lane 1, crude extract; lane 2, after DEAE-Sepharose; lanes 3 and 4, A and B forms after phenyl-agarose; lanes 5 and 8, size markers; lane 6, A-form after Phenyl-Superose and electroelution; lane 7, B-form after Phenyl-Superose.

1	2	3	4	5	6	7	8	9	10
Asp-Val-Gln-Gln-	?	-Asn-Ala-Glu-Asn-Ala-							
11	12	13	14	15	16	17	18	19	20
-Ser-Val-Arg-Lys-Glu-Trp-Gly-Ser-Leu-Thr-									
21	22	23	24	25	26	27	28	29	30
-Pro-Asp-Glu-Gln-Leu-Gly-Gly-	?	-	?	-Ala-					
31	32	33	34						
-Gly-Pro-	?	-Leu-							

Fig. 3. N-terminal amino-acid sequence of the B-form of AHTase.

Metal ion content

A sample of the B form of AHTase purified by ion-exchange and hydrophobic-interaction chromatography, and finally by fast-phase hydrophobic-interaction chromatography, was analysed for heavy metal content. Assuming a molecular mass for the B form of 48 kDa, it was calculated that the AHTase contains 2.1 copper and 0.9 zinc atoms per polypeptide. Chromium content was less than 0.02 atoms per polypeptide.

Enzyme properties

Assay with artificial substrate. In the colorimetric assay using HME as substrate (Fig. 1*b*), the maximum rate of colour development was not usually obtained immediately. This delay was minimized by the use of high substrate concentrations and the inclusion in the reaction mixture of catechol, as a primer for monophenol oxidation (Mayer & Harel, 1979). Activity measured by the colorimetric assay had a pH optimum of 7.0.

Estimates of the apparent K_m for HME in the presence of oxygen dissolved at atmospheric pressure, using crude or purified extracts, gave values ranging from 2 mM to 14 mM. Attempts to discover the source of this variation suggested that the purity of the enzyme had no effect, but the age of the substrate might be a factor: a fresh batch of HME initially proved a very poor substrate, but on storage, either as solid or in solution, gave higher activities and a lower K_m estimate.

The involvement of oxygen in the reaction was demonstrated by the lack of colour development in a nitrogen-flushed Thunberg tube, and by the use of an oxygen electrode.

High concentrations of ammonium ions ($K_a[NH_4^+] = 0.27$ M) in the assay mixture increased the rate of colour development four- to sixfold, but did not affect the K_m for HME. On the other hand, when assayed in the oxygen electrode, activity was only increased by 30%, suggesting that ammonium ions lead to enhanced colour development rather than affecting the primary oxidation steps. Removal of the added ammonium ions by dialysis returned the activity to the basal level. Ammonium ions also slightly accelerated the spontaneous oxidation to coloured products of both HME and catechol.

The conclusion that ammonium ions enhance secondary reactions of the oxidation products was supported by ascorbate delay assays. In these assays the primary oxidation products are re-reduced by ascorbate, and colour development is only seen when the ascorbate is exhausted. Although the eventual colour development was six times faster in the presence of ammonium ions than in their absence, the delay due to ascorbate was increased rather than decreased by ammonium ions, implying that the reaction products were less available for oxidation of ascorbate.

Methylammonium in place of ammonium ions gave a slight enhancement of apparent activity at 0.1 M, but was inhibitory at 1 M. Urea had no effect on reaction rates at 0.1 M, but was slightly inhibitory at 0.5 M.

The colorimetric assay was calibrated using both ascorbate delay and polarographic assays. In the absence of ammonium ions, an A_{470} of 1.0 in the colorimetric assay corresponded to the complete oxidation of 1.54 mM-ascorbate and to the incorporation of

Table 1. Purification of AHTase

	Total volume (ml)	Total protein (mg)	Total activity ($\Delta A_{470} \text{ s}^{-1}$)	Specific activity ($\Delta A_{470} \text{ s}^{-1} \text{ mg}^{-1}$)	Purification factor
Crude extract	205	47.15	8.05	0.17	(1.0)
DEAE-Sepharose	47	5.40	4.80	0.89	5.2
Phenyl agarose					
Form A	12	0.24	1.92	8.00	47.1
Form B	12	0.42	1.81	4.31	25.4

1.58 mM- O_2 . In the presence of 0.5 M-ammonium ions, an A_{470} of 1.0 was equivalent to 0.41 mM-ascorbate and 0.45 mM- O_2 .

Thermolability of AHTase from *ivoB192*. AHTase activity in crude mycelial extracts of *ivoB192* strains was more thermolabile than that in extracts of the wild-type obtained under the same conditions (Fig. 4). In addition, the activity of the mutant extract fell steeply during assay, giving only 48% of the initial activity after 4 min at 25 °C, whereas the wild-type activity over the same period increased by 32%.

The *ivoB192* enzyme differed from the wild-type in two further respects: ground mycelium was required for its detection, washings having very low activity; and activation by ammonium was 20- to 30-fold rather than 3- to 6-fold. In a mixed assay there was no evidence that addition of an *ivoB192* extract affected the stability of wild-type enzyme.

Fig. 4 shows that both mutant and wild-type enzymes show residual activity after heat treatment, suggesting that the enzyme is heterogeneous. However, electrophoresis in non-denaturing SDS gels of heat-treated wild-type enzyme showed that the activity of A and B forms declined in parallel. We were unable to repeat this test with the *ivoB192* extract because no activity was detectable after electrophoresis.

Oxidation of the natural substrate. The natural substrate is accumulated by *ivoB* mutants, which lack the phenol oxidase described in this paper (Clutterbuck, 1990). It has been purified in small quantities (McCorkindale *et al.*, 1983) and shown to be *N*-acetyl-6-hydroxytryptophan (AHT: Fig. 1*a*).

Purified AHT extracted from *ivoB* strains has been compared with the synthetic product (McCorkindale *et al.*, 1983). Both are substrates for the phenol oxidase as assayed polarographically and by destruction of substrate as determined with diazotized sulphanilic acid. Control extracts of *ivoB* strains failed to react with AHT in both tests.

A comparison of molecular extinction and response to the acid-diazotized sulphanilic acid test indicated that

Fig. 4. Thermolability of AHTase from *ivoB192*. Extracts were kept on ice and transferred for the period indicated to 60 °C. All extracts were then assayed at 25 °C. Each point represents a single test. Both extracts were from strains carrying additional markers *biA1* and *brlA7*. ○, *ivoB+*; ●, *ivoB192*.

Jepson's calibration of A_{530} of 1.0 as equivalent to 13 µg of 6-hydroxyindole (Jepson *et al.*, 1962) is applicable to AHT. Comparison of loss of substrate assayed in this way with oxygen utilization suggested that approximately two atoms of oxygen were absorbed per atom of pure substrate, but where unpurified natural substrate was used, up to four times as much oxygen was absorbed, and under these conditions oxygen absorption accelerated during the assay (a few minutes). Measurement of the K_m for AHT was unsuccessful due to the limited availability of natural or synthetic AHT and to the apparently non-linear kinetics in the one experiment attempted.

In vitro oxidation of purified AHT gave a brown solution, while crude methanol AHT extracts gave a blue

Table 2. Inhibitors of AHTase

Inhibitor	Inhibitor concn (mM)	Substrate concn (mM)	Inhibition (%)
2,3-Dihydroxynaphthalene	2.5	5	91
	10.0	5	97
	2.5	40	66
	10.0	40	87
Phenylhydrazine	1.0	67	86
Diethyl dithiocarbamate	0.1	67	100*
8-Hydroxyquinoline	3.0	67	44
<i>o</i> -Phenanthroline hydrate	3.0	67	30
Phenylthiourea	1.0	67	94
Sodium metabisulphite	0.1	67	99*
Sodium azide	3.0	67	0

* Inhibition declined with prolonged incubation: see text.

colour. Addition of purified AHT to slots in conidophore-bearing colonies of *ivoB*⁺ strains (but not *ivoB* strains) on agar also gave a blue coloration.

Other substrates. A variety of potential substrates were tested for colour reaction with *ivoB*⁺ strains using an *ivoB* strain as control. The following substances gave no coloration with either test strain: caffeic acid; *p*-coumaric acid; dihydroquercetin; hydroquinone monobenzyl ether; 5-hydroxytryptophan; 4-*o*-methyl-orcellinic acid; α -naphthol; quercetin; resorcinol; rutin; tyrosine; while the following gave similar coloration with both test strains: *p*-tertiary-butylcatechol; D-catechin; catechol; chlorogenic acid; 2,6-dimethoxyphenol; dihydroxyphenylalanine; aesculin hydrate; hydroquinone; *o*-methoxyphenol (guaiacol); 3-methylcatechol; 4-methylcatechol; tannic acid.

The following gave slightly more coloration with wild-type or *ivoA* strains than with *ivoB* strains: *p*-cresol; 3,4-dihydroxyphenylacetic acid; *p*-hydroxydiphenyl; *m*-methoxyphenol. The last of these compounds was also tested in the polarographic assay, but no oxidation was detected.

5-Hydroxytryptophan, 6-hydroxytryptamine and *N*-acetyl-6-hydroxytryptophan methyl ester were also tested polarographically, and the latter two were tested for loss of diazotized sulphanilic acid staining after treatment with the oxidase, but none showed significant activity.

Inhibitors. Table 2 gives a list of inhibitors acting on the colorimetric assay. 2,3-Dihydroxynaphthalene is a competitive inhibitor of the cresolase reaction (Mayer *et al.*, 1964), and kinetic data (not shown) indicated that inhibition of AHTase was also competitive, although the kinetics were not strictly linear. Likewise phenylhydrazine is a non-competitive inhibitor of AHTase and of catechol oxidase (Mayer *et al.*, 1964). The remaining

inhibitors are believed to act by reacting with the copper component of the enzyme. Inhibition by sodium metabisulphite and diethyl dithiocarbamate declined with prolonged incubation, and in the latter case a white precipitate developed. This suggests that these compounds are incomplete inhibitors and can be destroyed by the primary products of the reaction.

Inhibition of the oxidase *in vivo* for colonies grown on 2 mM-sodium metabisulphite led to the accumulation of material which stained as a 6-hydroxyindole with diazotized sulphanilic acid. This method has been used to test for the presence of this substrate in a variety of *Aspergillus* species (A. J. Clutterbuck, unpublished).

Discussion

The enzyme described here has an unusual natural substrate, but in other respects its properties suggest that it is a monophenol monooxygenase (EC 1.14.18.1) (Bell & Wheeler, 1986; Mayer & Harel, 1979; Mayer *et al.*, 1964). While we have no evidence that AHTase catalyses the oxidation of the standard substrate tyrosine or catechol, it does oxidize HME and *p*-cresol and is inhibited by a number of typical phenol oxidase inhibitors.

The presence of copper in this enzyme is also typical of phenol oxidases (Fling *et al.*, 1963) and we have evidence from the *ygA* mutant, which is believed to limit the intracellular availability of copper (Clutterbuck, 1972, 1990), that this metal is required for activity.

On the other hand the finding of zinc in AHTase is unexpected and we have no evidence of its function. Its presence, however, may imply a relationship with other cuprozinc proteins such as superoxide dismutase (Fridovich, 1975) and cytochrome oxidase (Moubarak *et al.*, 1987).

The observation that there are two forms of the enzyme separable by hydrophobic interaction chromatography and SDS-PAGE is not unusual for phenol oxidases. In this case we have detected no difference in activity or prevalence of the A and B forms. They share a common N-terminus and differ in one out of three peptides. Both forms bind to concanavalin A (Birse, 1989), which implies that they are glycoproteins, so it is possible that the difference between them lies in the degree of glycosylation, as in *Podospira anserina* laccase II (Esser & Minuth, 1971). Another possibility is that the smaller isoenzyme has suffered partial proteolytic degradation, but if so this has neither destroyed its activity nor affected the N-terminal residues. The presence of a variable lipid C-terminal anchor (Ferguson & Williams, 1988) is a third possibility.

The enzyme was fully active on extraction, and there

was no evidence of the latency found with other phenol oxidases (Mayer & Harel, 1979). Enhancement of activity by ammonium ions is clearly not an enzyme-activation process, but a modification of secondary reactions leading to pigment formation, and hence to a fivefold increase in activity in the colorimetric assay. The 30% increase in oxygen utilization in the presence of ammonium ions can be attributed to more efficient product removal. It is interesting that activity of extracts of the *ivoB192* mutant were more sensitive to ammonium ions than wild-type extracts; if ammonium ions affect only the later stages of melanin formation, this implies that the enzyme is involved here as well as in oxidation of the initial substrate.

The enzyme is remarkably resilient. It maintains its activity for days at room temperature, and is extractable as an extracellular product of conidiating cultures grown for 5 d at 37 °C. Cultures of this age produce extracellular protease (Cohen, 1977; A. J. Clutterbuck, unpublished) to which the enzyme is evidently resistant. The enzyme is also readily purified from extracellular extracts, in which it appears to constitute at least 1% of the total protein, although the conidial apparatus which produces it forms only a minority of the mycelium harvested.

Our main interest in this enzyme is that it is an identifiable biochemical step in the conidial development of *A. nidulans*. The *ivoB* gene is known from biological evidence (Clutterbuck, 1969, 1990) and from molecular analysis (Adams *et al.*, 1988) to be under control of the *brlA* gene. In this paper we have given evidence, in terms of the thermolability of AHTase from the *ivoB192*, mutant that *ivoB* is the structural gene for this enzyme. We have confirmed this (Birse & Clutterbuck, 1990) by using the N-terminal amino acid sequence to construct a DNA probe which hybridizes with an *ivoB* clone isolated by mutant complementation. We now plan to use the *ivoB* clone to investigate the nature of its developmental control by *brlA*.

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