Characterization of Purine Hydroxylase I from *Aspergillus nidulans*

By RAJ K. MEHRA¹ AND MICHAEL P. COUGHLAN²*

¹The Agricultural Institute, Moorepark Research Centre, Fermoy, Co. Cork, Ireland

²Department of Biochemistry, University College, Galway, Ireland

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Purine hydroxylase I from *Aspergillus nidulans* was purified 850-fold. The purified preparations exhibited the spectral and catalytic properties, including broad specificity for oxidizing and reducing substrates, typical of molybdenum/flavin/iron-sulphur-containing hydroxylases (oxotransferases).

INTRODUCTION

Molybdenum-containing hydroxylases are widely distributed among prokaryotic and eukaryotic species (Krenitsky *et al.*, 1974; Wurzing & Hartenstein, 1974; Coughlan, 1980*a*). These enzymes, which may more properly be termed oxotransferases (Berg & Holm, 1985*a, b*), were until quite recently thought to include only aldehyde oxidase, nitrate reductase, xanthine oxidase and xanthine dehydrogenase. However, in the last twenty years the number of enzymes identified as belonging to this group has increased to about fifteen (e.g. Massey, 1973; Coughlan, 1980*b*, 1983; Friedrich *et al.*, 1986; Hinojosa-Leon *et al.*, 1986; Turner *et al.*, 1987; Bell *et al.*, 1988). They are unique in that they all possess a molybdopterin cofactor (Johnson, 1980; Johnson & Rajagopalan, 1982). Among the enzymes in this group are the purine hydroxylases I and II from *Aspergillus nidulans*. These have been submitted to thorough genetic analysis (Lewis *et al.*, 1978; Scazzocchio, 1980; Scazzocchio & Sealy-Lewis, 1978; Scazzocchio *et al.*, 1973). However, while some properties of purine hydroxylase I have been examined using crude extracts (Lewis *et al.*, 1978), only purine hydroxylase II has been purified and subjected to extensive biochemical characterization and electron paramagnetic resonance spectroscopy (Coughlan *et al.*, 1984; Mehra & Coughlan, 1984). We report here some of the biochemical properties of purified purine hydroxylase I.

METHODS

Organism. The biotin-requiring *Aspergillus nidulans* strain biA-1 used in these studies was kindly provided by Professor Claudio Scazzocchio, Université Paris-Sud, Orsay, France. The organism was grown as described previously using 2-thioxanthine (0.5 mg l⁻¹) to induce synthesis of purine hydroxylase I (Scazzocchio *et al.*, 1973).

Electrophoresis. Protein purity and *M_r* values were estimated by polyacrylamide gradient gel electrophoresis using the procedures recommended by Pharmacia Fine Chemicals (Publication no. 11-B-037-11, August 1978). The gel gradient was 5–27% and the buffer was 70 mM-Tris, 80 mM-boric acid, 2.5 mM-disodium EDTA, pH 8.3. Standard proteins were those supplied by Pharmacia as the high-molecular-weight calibration kit. Gels were fixed in 10% (w/v) trichloroacetic acid and then stained for protein in 0.2% (w/v) gel electrophoresis blue 83 in water/methanol/acetic acid (6:3:1, by vol.) and destained in water/methanol/acetic acid (6:3:1, by vol.) until the background was clear. Replicate gels were stained for activity using a mixture of hypoxanthine (450 µM) and either nitroblue tetrazolium (1.27 mg ml⁻¹) or *p*-iodonitrotetrazolium (3 mM) in 0.1 M-sodium pyrophosphate buffer, pH 7.8, containing 0.2 mM-EDTA. The gels were scanned densitometrically at 550 nm (protein or activity stains) by using a Pye Unicam SP1800 spectrophotometer specially adapted for that purpose.

Isoelectric focusing. This was done using Pharmalytes covering the pH range 3–10, the procedure and the isofocusing standard proteins recommended by Pharmacia Fine Chemicals (Publication no. 01-900-2-1464-02). Gels were stained for protein or activity as above and pI values were determined by comparison with those of standards.

Enzyme assays. Reactions were carried out at 25 °C in 100 mM-sodium pyrophosphate buffer, pH 7.8, containing 200 μ M-EDTA in a final volume of 1.0 ml. The oxidation of hypoxanthine (450 μ M) by the following acceptors, their concentrations, and the wavelengths at which reactions were monitored, were as follows: oxygen (i.e. air-saturated buffer, 2.3 mM; 278 nm); 2,6-dichlorophenolindophenol (50 μ M; 600 nm); trinitrobenzene sulphate (0.5 mM; 278 nm); phenazine methosulphate (0.1 mM; 278 nm); methylene blue (20 μ M; 278 nm); NAD⁺ (2.26 mM; 340 nm); ferricyanide (2 mM; 420 nm). The molar absorption coefficient differences at the appropriate wavelengths between substrate and product (or oxidized and reduced acceptor) were: hypoxanthine, 8333 l mol⁻¹ cm⁻¹; ferricyanide, 1040 l mol⁻¹ cm⁻¹ (since reaction was monitored at 420 nm allowance was made for the fact that for every 1 mol of hypoxanthine oxidized 2 mol of ferricyanide were reduced); 2,6-dichlorophenol indophenol, 21 000 l mol⁻¹ cm⁻¹; and NAD⁺, 6220 l mol⁻¹ cm⁻¹. For routine purposes 1 unit of activity is defined as being equivalent to 1 μ mol NADH produced min⁻¹. The oxidation of NADH (450 μ M) by ferricyanide was followed at the appropriate wavelength.

Protein determination. Protein concentration in crude extracts was measured by the method of Hartree (1972) using bovine serum albumin as standard. Enzyme concentration in purified preparations was measured using the previously determined ϵ_{450} value for purine hydroxylase II of 72 mm⁻¹ cm⁻¹ (Mehra & Coughlan, 1984).

Enzyme purification. All procedures, unless stated otherwise, were carried out at 4 °C using 100 mM-sodium pyrophosphate buffer, pH 7.8, containing 200 μ M-EDTA.

Preparation of crude extract. Frozen cells were thawed in 4 vols buffer and homogenized for 3 min in a 5 litre Waring blender with an equal volume of glass beads (0.25–0.31 mm), the temperature being maintained below 10 °C during breakage. The homogenate was centrifuged at 2000 g for 30 min using a Mistral 6 L centrifuge. The supernatant, hereinafter called the crude extract, was collected. The enzyme activity in such extracts was very unstable but was stabilized somewhat by ammonium sulphate fractionation as described below.

Ammonium sulphate fractionation. Ammonium sulphate (final concentration 30% saturation) was added slowly to the crude extract with stirring, the pH being maintained at 7.8 using NaOH. Stirring was continued for 30 min after the last addition of salt. Following centrifugation the supernatant was collected and ammonium sulphate was added to it to give 55% saturation. Following equilibration and centrifugation the precipitate was collected, resuspended in 4 vols (w/v) 50 mM-Tris/HCl buffer, pH 7.8, containing 0.2 mM-EDTA and clarified by centrifugation at 27 000 g for 30 min. The pH of the supernatant, which was collected, was adjusted to 7.8 when necessary.

Acetone fractionation. Acetone, at –20 °C, was added dropwise with stirring to the above supernatant to a final concentration of 50% (v/v), the temperature being maintained between –5 and 0 °C using a salt-ice bath. The mixture was stirred for 10 min after the last addition of acetone and then centrifuged for 1 min at 20 000 g at –10 °C. The precipitate was resuspended in 4 vols 50 mM-Tris/HCl buffer, pH 7.8, containing 0.2 mM-EDTA, and centrifuged at 27 000 g for 30 min. The enzyme solution was concentrated by precipitation with ammonium sulphate (50%, w/v) and redissolving in a small volume of 50 mM-Tris/HCl buffer, pH 7.8, containing 0.2 mM-EDTA.

Gel filtration. Samples (3.2 ml) of enzyme from the previous step were applied to a Sephadex G-200 column (2.5 × 59 cm; bed volume 290 ml) pre-equilibrated with 50 mM-Tris/HCl buffer, pH 7.8, containing 0.2 mM-EDTA. Flow rate was set at 10 ml h⁻¹ and 2 ml fractions were collected. Protein was eluted by irrigation with the same buffer. Enzyme activity eluted as a single symmetrical peak. Fractions of high specific activity were pooled. Activity was relatively stable at this stage. In subsequent runs Sephadex G-200 was replaced by Sephacryl-300 because of ease of packing and better flow rates. In this case 10 ml samples of enzyme solution from the ammonium sulphate step were applied to a 1 litre column of Sephacryl-300 pre-equilibrated with 50 mM-Tris/HCl buffer, pH 7.8, containing 0.2 mM-EDTA.

Ion-exchange chromatography. The pooled enzyme from the previous step was applied to a Whatman DE 52 cellulose column (0.7 × 13.0 cm; bed volume 5 ml) equilibrated with 50 mM-Tris/HCl buffer, pH 7.8, containing 0.2 mM-EDTA. The column was then irrigated with this buffer containing 0.1 M-NaCl until protein no longer eluted. A linear gradient (150 ml) of 0.1 to 0.4 M-NaCl, in the above buffer, was then applied at a flow rate of 20 ml h⁻¹. Fractions (1.65 ml) were collected. Enzyme activity was eluted at 0.13–0.16 M-NaCl. Fractions of highest specific activity were pooled.

RESULTS AND DISCUSSION

The purification scheme outlined above and summarized in Table 1 resulted in an 850-fold purification of purine hydroxylase I with a yield of about 9%.

Properties of purine hydroxylase I

Absorption spectrum. The absorption spectra of hydroxylases containing molybdenum, flavin and iron-sulphur prosthetic groups are characterized by peaks at 280 nm and 450 nm (the latter

Fig. 1. Visible absorption spectrum of purine hydroxylase I.

Table 1. *Purification of purine hydroxylase I*

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity [units (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude extract	7010	35729	50.1	0.0014	1	100
Ammonium sulphate (30–55% cut)	940	5541.3	39.1	0.0071	5	78
Acetone (0–50% cut)	240	236.9	35.5	0.15	107	70.9
Sephadex G-200	21.5	17.1	8.3	0.486	347	16.6
Cellulose DE 52	40	3.9	4.6	1.19	850	9.2

being due mainly to the flavin chromophores) and a shoulder at 550 nm reflecting the absorption by the iron-sulphur centres (Coughlan, 1980*a*). Purified preparations of such enzymes have A_{280}/A_{450} ratios of 5 and A_{450}/A_{550} ratios of about 3 (Rajagopalan & Handler, 1964; Coughlan, 1980*a*). The visible absorption spectrum of purified purine hydroxylase I showed the expected absorption at 450 nm and the characteristic shoulder at 550 nm (Fig. 1). The A_{450}/A_{550} ratio of the preparation shown was 2.7. Thus, purine hydroxylase I is typical of that group of molybdenum/flavin/iron-sulphur enzymes that contain 2(2Fe–2S) centres per mol flavin (Rajagopalan & Handler, 1964). The visible spectra revealed that the enzyme preparations obtained using the procedure outlined above were not contaminated by coloured protein (Coughlan, 1980*a*). However, variation of the A_{280}/A_{450} ratios of several purine hydroxylase I preparations between 9 and 11 indicated that such preparations were contaminated with colourless protein (see below).

Electrophoretic properties. Polyacrylamide gradient gel electrophoresis of a typical preparation demonstrated the presence of two protein bands, only one of which stained for activity. The M_r value of the contaminating protein was between 386 000 and 400 000 while that of the purine hydroxylase I band was calculated to be between 260 000 and 280 000. This is slightly lower than the usual value of about 300 000 for such enzymes (Coughlan, 1980*a*) and the value of 304 000 reported for purine hydroxylase I in crude extracts (Lewis *et al.*, 1978) but is in agreement with the value of 260 000–280 000 for partially purified preparations of this enzyme (H.M. Sealy-Lewis & C. Scazzocchio, personal communication). These workers found that proteolysis occurring, without loss of activity, during isolation was responsible for the decrease in the M_r value of the native enzyme. In view of this finding and since hydrolysis of molybdoenzymes by endogenous proteases during isolation is a common observation (see e.g. Waud *et al.*, 1975), we

Table 2. *Electron acceptor utilization by purine hydroxylase I*

In all cases hypoxanthine (450 μM) was the reducing substrate. Catalytic centre activity is expressed as mol substrate oxidized (mol enzyme subunit) $^{-1}$ min $^{-1}$. Enzyme subunit concentration was calculated from the A_{450} of the enzyme solution and a subunit ϵ_{450} value of 36 mm $^{-1}$ cm $^{-1}$ (Coughlan, 1980a; Mehra & Coughlan, 1984).

Electron acceptor	Apparent K_m (μM)	Catalytic centre activity
Ferricyanide	2000.0	662
Phenazine methosulphate	7.0	935
Dichlorophenolindophenol	45.5	546
Trinitrobenzene sulphonate	540.0	443
Methylene blue	190.0	354
NAD $^{+}$	331.0	342
O $_2$	ND	0.8

ND, Not determined.

may presume that the purine hydroxylase I preparations isolated here have also been subject to proteolysis. The most dramatic consequence of such hydrolysis of rat liver xanthine dehydrogenase is that its ability to utilize NAD $^{+}$ as an electron acceptor is greatly decreased while the utilization of oxygen is greatly increased; i.e. the enzyme is converted from a dehydrogenase to an oxidase (Waud & Rajagopalan, 1976a, b). Such is not the case with purine hydroxylase I, the enzyme being a true dehydrogenase throughout the purification process (see below).

Densitometric scanning of electrophoretic gels in the present study suggested that purine hydroxylase I accounted for at least 60% of the total protein of the purified preparations. On isoelectric focusing, only one protein band, which also stained for activity, was obtained, indicating that both purine hydroxylase I and the contaminating protein had a pI value of 5.0. Procedures used in attempts to obtain further purification of purine hydroxylase I included calcium phosphate gel chromatography, chromatofocusing, preparative polyacrylamide gel electrophoresis and affinity chromatography on Sepharose 4B-folate (Nishino *et al.*, 1982) or Blue-Sepharose gels. Such procedures have proven successful with the corresponding enzymes from other sources but did not prove to be so in the case of purine hydroxylase I.

Specificity for electron acceptors. Purine hydroxylase I, as isolated, is a true dehydrogenase in that it utilizes NAD $^{+}$ rather than oxygen in the hydroxylation of substrates. Thus, with hypoxanthine (450 μM) as reducing substrate, the catalytic centre activity [i.e. mol substrate oxidized (mol enzyme subunit) $^{-1}$ min $^{-1}$] with oxygen as oxidizing substrate was only 0.8 while that with NAD $^{+}$ was 342. The apparent K_m value for the latter, the probable acceptor *in vivo*, was 331 μM . As is the case with all of the molybdenum hydroxylases isolated to date, purine hydroxylase I could catalyse the transfer of electrons to a variety of artificial acceptors. The apparent K_m values and catalytic centre activities with such acceptors are given in Table 2.

Specificity for reducing substrates. Purine hydroxylase I was able to catalyse the oxidation of NADH and the hydroxylation of a wide variety of purines, pyrimidines, pteridines and aldehydes, albeit at different rates (Table 3). In that it has broad reducing substrate specificity, purine hydroxylase I is similar to all other xanthine oxidases, xanthine dehydrogenases and aldehyde oxidases examined to date (Krenitsky *et al.*, 1972; Coughlan, 1980a). The ability to catalyse the hydroxylation of xanthine and the inability to act on nicotinic acid distinguish it from purine hydroxylase II from the same organism (Lewis *et al.*, 1978; Mehra & Coughlan, 1984). The relative rates of action of the purified purine hydroxylase I upon hypoxanthine, xanthine and nicotinic acid are in agreement with the results obtained using crude extracts of *A. nidulans* biA-1 (Lewis *et al.*, 1978). We concur with the conclusion of these authors that, *in vivo*, purine hydroxylase I is primarily engaged in purine metabolism and purine hydroxylase II in nicotinate metabolism.

A number of points with regard to substrate specificity are deserving of further comment. The fact that adenine, 6-mercaptopurine, 6-methoxypurine and 6-thioxanthine were not acted upon

Table 3. Reducing substrate specificity of purine hydroxylase I

Except where stated otherwise, the final reducing substrate concentration was 450 μ M. NAD⁺ (2.26 mM) was the acceptor in all but one case. Activities are expressed as a percentage of that given with hypoxanthine as substrate.

Substrate	Relative rate	Apparent K_m (μ M)
Hypoxanthine (6-hydroxypurine)	100.0	16.4
Adenine (6-aminopurine)	0.7	
6-Mercaptopurine	1.8	
6-Methoxypurine	1.8	
Xanthine (2,6-dihydroxypurine)	61.6	34.2
2-Thioxanthine (2-thio-6-hydroxypurine)	28.6	15.6
6-Thioxanthine (2-hydroxy-6-thiopurine)	2.1	
8-Chloroxanthine (2,6-dihydroxy-8-chloropurine)	0	
Purine	21.8	10.7
2-Hydroxypurine	42.9	16.8
Nicotinic acid	0.6	
<i>N</i> ¹ -Methylnicotinic acid	0.2	
<i>N</i> ¹ -Methylnicotinamide	0	
4-Hydroxypyrazolo[3,4- <i>d</i>]pyrimidine (allopurinol)	7.6	
4-Aminopyrazolo[3,4- <i>d</i>]pyrimidine	0.8	
4-Mercaptopyrazolo[3,4- <i>d</i>]pyrimidine	0.4	
2-Amino-4-hydroxypteridine (pterin)	19.8	
2-Amino-4,6-dihydroxypteridine	8.1	
Folic acid	0.4	
Benzaldehyde (4.3 mM)	24.1	1370.0
Formaldehyde (30 mM)	6.2	
Acetaldehyde (30 mM)	0.5	
NADH (2 mM-ferricyanide as acceptor)	115.3	63.5

suggests that substitutions at position 6, other than -H (purine) or -OH (hypoxanthine), prevented productive binding to the active site. Substitution of the -H at position 8 (as in xanthine) or by -Cl (8-chloroxanthine) completely abolished activity. By contrast, substitution at position 2 had relatively little effect. Thus, 2-thioxanthine was converted to 2-thiouric acid at half the rate of conversion of xanthine to uric acid while 2-hydroxypurine was acted upon at twice the rate of utilization of purine. The finding that 2-hydroxypurine did not support growth of the organism whereas xanthine (2,6-dihydroxypurine) did indicates that 2-hydroxypurine is hydroxylated at position 8 rather than at position 6 (Scazzocchio & Sealy-Lewis, 1978). The inability to utilize *N*¹-methylnicotinamide at pH 7.8 is not surprising. Purine hydroxylase II (Mehra & Coughlan, 1984), milk xanthine oxidase (Greenlee & Handler, 1964) and turkey liver xanthine dehydrogenase (Cleere & Coughlan, 1975) cannot act on this substrate at physiological pH but can do so at higher pH values. Greenlee & Handler (1964) interpreted such findings as indicating the presence at the active centre of xanthine oxidase of a residue, possibly lysine, with a pK of 10.7. In the protonated form this would repel the positively charged substrate. In the case of milk xanthine oxidase the K_m values for aldehyde substrates are of the order of millimolar or greater. The situation with respect to purine hydroxylase I is clearly the same. Finally, we note that purine hydroxylase I, like the avian xanthine dehydrogenases (Coughlan, 1980*a*), readily catalyses the oxidation of NADH.

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