EFFECTS OF ALLOPURINOL AND OF OXYPURINOL ON TURKEY LIVER XANTHINE DEHYDROGENASE

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1. Introduction

Allopurinol a potent inhibitor of xanthine:oxygen oxidoreductase (EC 1.2.3.2) activity of milk xanthine oxidase is converted to oxypurinol by the enzyme [1]. Enzyme incubated with allopurinol rapidly loses the ability to catalyse the oxidation of xanthine by O_2. By contrast preliminary incubation with oxypurinol causes no decrease in activity unless xanthine is also present [2]. Inactivation results from complex formation between oxypurinol and the enzyme-bound molybdenum in the tetravalent state [3,4]. The effects of allopurinol and oxypurinol on the xanthine dehydrogenases (purine hydroxylases) from *Aspergillus nidulans* differ considerably from those on xanthine oxidase (Scazzocchio, personal communication; Lewis, Hurt, Sealy-Lewis and Scazzacchio, in preparation). Work with crude extracts showed both agents to be pseudo-irreversible inhibitors of purine hydroxylase I. However, inactivation by allopurinol appears not to be due to its conversion to oxypurinol. With purine hydroxylase II, allopurinol is a competitive inhibitor while oxypurinol shows anti-competitive inhibition. In this case neither compound forms an inactive complex with the enzyme. In order to determine whether these findings are unique to the enzymes from *Aspergillus* or shared by xanthine dehydrogenases generally we decided to examine the effects of allopurinol and oxypurinol on the highly purified xanthine dehydrogenase (EC 1.2.1.37) from turkey liver.

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2. Materials and methods

Fresh-frozen turkey livers were obtained from Castlemahon Co-op, Newcastle West, Co. Limerick and stored at −20°C until required. Enzyme having the full compliment of molybdenum, flavin and iron–sulphur groups, was isolated from this source by a modification [6] of the method in [7]. Samples of different degrees of functionality (see below) were prepared by mixing native enzyme and cyanide-treated enzyme in the appropriate proportions [8].

All reactions were carried out at 30°C in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA, in final vol. 1.0 ml. The oxidation of xanthine (150 μM) by the following acceptors and the wavelengths at which reactions were monitored were as follows: 2,6-dichlorophenolindophenol (50 μM, 600 nm); trinitrobenzene–sulphonate (500 μM, 295 nm); methylene blue (20 μM, 295 nm); O_2 (air-saturated buffer, 295 nm); ferricyanide (2.0 mM, 420 nm); NAD^+ (500 μM, 340 nm). The oxidation of NADH (50 μM) by the above acceptors (with the exception of 2,6-dichlorophenolindophenol) was followed at 340 nm. Xanthine:NAD^+ oxidoreductase activity is expressed as the activity:flavin ratio, obtained by dividing the change in A_{340}/min by the A_{450} of the enzyme used in the assay. Fully functional enzyme has a value of 325 under these conditions [8].

Enzyme active site concentration (2 active sites/molecule) was determined from the ϵ_{450} value of 36 550 litre mol^{-1} cm^{-1} [7]. Allopurinol and oxypurinol concentrations were determined from the molar extinction coefficient values, at pH 8.3. These

are 7800 litre mol^{-1} cm^{-1} (at 250 nm) and 8250 litre mol^{-1} cm^{-1} (at 242 nm), respectively [3–5].

Bleaching of the visible absorption spectrum of the enzyme by allopurinol was followed at 25°C using a Cary 118 spectrophotometer. The anaerobic cuvettes used were supplied by Hellma (England), Westcliff-on-Sea, Essex. The enzyme solution was placed in the side-arm and the buffered substrate in the centre well was flushed with O_2-free nitrogen for 15 min before mixing. Reaction conditions are described in the text.

The initial fast phase of reduction of enzyme by allopurinol was monitored at 450 nm using an Applied Photophysics stopped-flow device. The drive syringes and the mixing chamber were maintained at 25 ± 0.1°C. Kinetic data were recorded with a Tectronix model 5100 storage oscilloscope. The stored traces were photographed and used to determine first-order rate constants. Reaction conditions are described in the text.

3. Results and discussion

Incubation of turkey liver xanthine dehydrogenase with allopurinol at 30°C resulted in the progressive loss of xanthine:NAD^+ oxidoreductase activity. Less than 1.0% of the original activity remained after 3 min. A similar rapid loss of activity was observed when enzyme was preincubated with oxypurinol in the presence of xanthine. However, enzyme incubated with oxypurinol in the absence of xanthine retained 80% of its original activity after 10 min. These results confirm those in [1] using milk xanthine oxidase (see section 1).

Evidence has been presented [3,4] showing that inactivation by these agents resulted from the binding of oxypurinol to a reduced form of the enzyme (see also [9]) and specifically to the enzyme-bound molybdenum in the Mo(IV) state. This, by preventing the uptake of electrons from substrates such as xanthine which interact with the molybdenum locus [5,10], explains the inactivation by allopurinol and by oxypurinol (in the presence of xanthine) of all xanthine-oxidizing activities of the turkey liver enzyme (table 1). It also explains the failure of these agents to prevent the enzyme-catalyzed oxidation of NADH, which interacts with the enzyme-bound flavin, by methylene blue, ferricyanide or O_2 (table 1). The

Table 1
Inactivation of various oxidoreductase activities of turkey liver xanthine dehydrogenase by allopurinol and by oxypurinol

Oxidoreductase activity	Additions	
	Allopurinol	Oxypurinol + xanthine
Xanthine : dichlorophenol-indophenol	24.7	21.3
Xanthine : trinitrobenzene sulphonate	5.4	7.9
Xanthine : methylene blue	0	0
Xanthine : ferricyanide	0	0
Xanthine : O_2	0	0
Xanthine : NAD^+	0	0
NADH : dichlorophenol-indophenol	35.3	35.8
NADH : trinitrobenzene sulphonate	14.8	16.1
NADH : methylene blue	100.0	100.6
NADH : ferricyanide	102.0	116.0
NADH : O_2	111.8	100.0

Enzyme samples (approx. 1.0 μM) were incubated at 30°C in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 0.25 mM allopurinol or 0.25 mM oxypurinol (plus 0.15 mM xanthine; cf. section 2). Aliquots were taken after 5 min for assays. The values given are specific activities based on the A_{450} and are in each case expressed as a percentage of those of the untreated enzyme

failure of allopurinol to affect the NADH:ferricyanide oxidoreductase activity of milk xanthine oxidase has been reported [3,4].

We have presented evidence that the electron acceptors dichlorophenol indophenol and trinitrobenzene sulphonate interact with the molybdenum centres of the turkey enzyme [11]. Accordingly, displacement of the molybdenum-bound oxypurinol by these electron acceptors may explain why the oxidation of xanthine by dichlorophenol indophenol and by trinitrobenze sulphonate is not completely inactivated (table 1). Competition between acceptor and inhibitor also explains why of the NADH oxidoreductase activities only those utilising dichlorophenol indophenol and trinitrobenzene sulphonate are affected (table 1).

The reactivation of oxypurinol-inactivated xanthine oxidase following prolonged incubation with phenazine methosulphate, ferricyanide or O_2 has been

observed [3,4]. This presumably reflects the slow reoxidation of the inactivated enzyme-bound molybdenum from the Mo(IV) to the Mo(VI) state with the concomitant release of bound oxypurinol. Preliminary results suggest that similar reactivation of oxypurinol-inactivated turkey enzyme occurs. However, and presumably for the reasons outlined earlier, dichlorophenol indophenol and trinitrobenzene sulphonate appear to be the most effective in this regard.

Figure 1 shows, as expected of a reducing substrate, that allopurinol bleaches the visible absorption spectrum of the turkey enzyme. The decrease in A_{450} and A_{550} and the increase in A_{610} shows that the flavin and iron–sulphur chromophores as well as the molybdenum centres are reduced. Stopped-flow

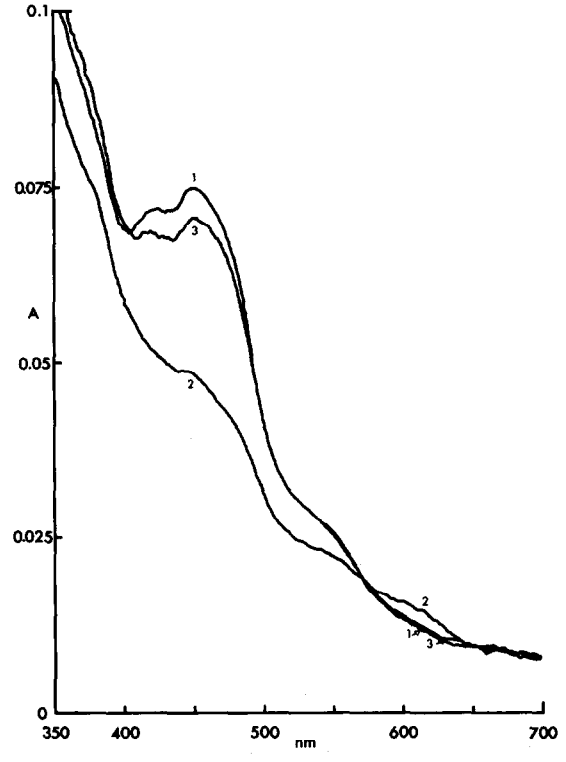

Fig.1. Effect of allopurinol on the visible absorption spectrum of turkey liver xanthine dehydrogenase. Curve 1, native enzyme (2.0 μM with respect to flavin) in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA, 25°C. Curve 2, spectrum recorded 30 s after mixing the above enzyme with 75 μM allopurinol. Curve 3, spectrum recorded 30 min after admission of air to allopurinol-inactivated enzyme.

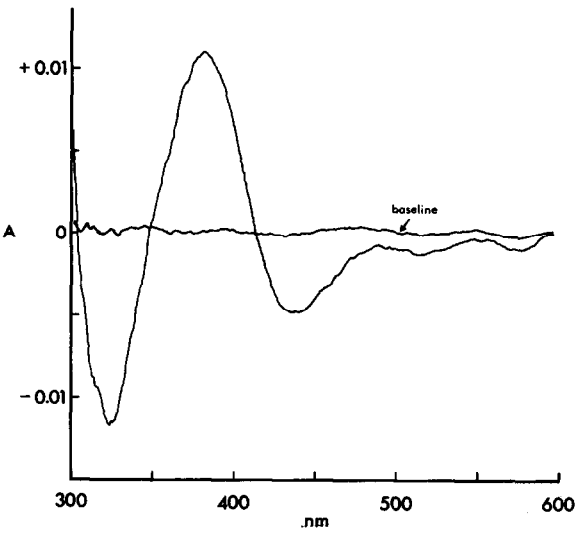

Fig.2. Difference spectrum between allopurinol-treated and native turkey liver xanthine dehydrogenase. Difference spectrum recorded between allopurinol-treated enzyme, 4.3 μM with respect to flavin, following reoxidation (curve 3, fig.1) in the sample compartment and native enzyme at the same concentration (curve 1, fig.1) in the reference compartment.

studies showed the pseudo first-order rate constant for the rapid phase of reduction (in which 4 electrons are taken up [5,6]) to be 63 ± 10 s^{-1} at 25°C, pH 7.8, and at an allopurinol to flavin ratio of 20:1. On admission of air the original absorption does not return, rather a modified spectrum is produced. Figure 2 shows that the difference spectrum between allopurinol-inactivated enzyme (presumably representing the complex between oxypurinol and enzymic molybdenum) and native enzyme is characterized by troughs at 320 nm and 440 nm and a peak at 380 nm. Figure 3 shows that the extent of the ΔA_{380} between inactivated enzyme and native enzyme is directly proportional to the content of functional active sites in the enzyme samples used. Non-functional enzyme lacks the cyanolysable sulphur atoms at the molybdenum loci necessary for the hydroxylation of substrate [8,12–14] and hence for the conversion of allopurinol to oxypurinol. Accordingly, the proportionality between the ΔA_{380} and enzyme functionality is not surprising. Figure 4 illustrates an experiment on the stoicheiometry of oxypurinol inactivation of the

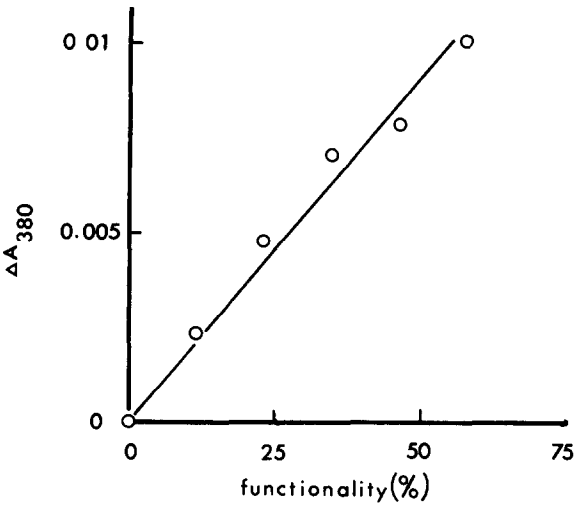

Fig.3. Correlation between the extent of spectral change on allopurinol treatment and the degree of functionality of the enzyme. Enzyme samples (approx. 4.0 μM with respect to flavin) differing in the content of functional active sites were treated anaerobically with 75 μM allopurinol as in fig.1. Following reoxidation the extent of ΔA_{380} was measured against the appropriate untreated sample.

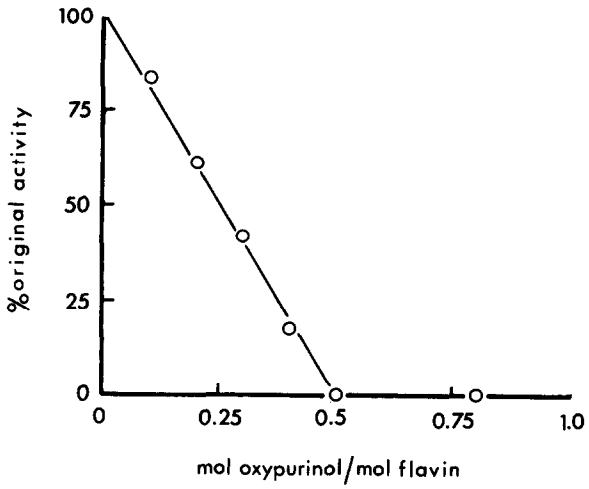

Fig.4. Stoicheiometry of oxypurinol inactivation of turkey liver xanthine dehydrogenase. Enzyme samples (1.56 μM with respect to flavin and 49% functional) were incubated under anaerobic conditions at 25°C for 30 min in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 M EDTA, 75.0 μM xanthine and the molar proportions of oxypurinol shown. Following reoxidation aliquots were taken for assay of xanthine:NAD^+ oxidoreductase activity. The values obtained are expressed as a percentage of that of enzyme treated as above except that oxypurinol was excluded.

turkey enzyme. It can be seen that inactivation was complete at an oxypurinol:active site ratio of 0.5:1.0. However, the enzyme sample used in this experiment was only 49% functional initially indicating that for fully functional enzyme complete inactivation would result from the binding of 1.0 ml oxypurinol/active centre (i.e., 2 mol/mol enzyme).

The results presented here show that the effects of allopurinol on turkey liver xanthine dehydrogenase are qualitatively and quantitatively similar to those reported for xanthine oxidase [1–4,9,13]. Thus, it would appear that the effects of these agents on the related enzymes from *Aspergillus nidulans* (see section 1) are unique. If and when these studies are extended to the purified hydroxylases from this organism we should obtain much valuable information on the structures of these most complex of enzymes.

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