Kinetic Studies of the Phenol Sulphate–Phenol Sulphotransferase of *Aspergillus oryzae*

By GARY R. J. BURNS, ELIZABETH GALANOPOULOU and COLIN H. WYNN
Department of Biochemistry, University of Manchester, Manchester M13 9PL, U.K.

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Arylsulphatase II of *Aspergillus oryzae* exhibits both hydrolytic and sulphotransferase activities. The kinetic data suggest the formation of an intermediate covalent enzyme–sulphate complex with transfer of sulphate from donor to acceptor proceeding via a Ping Pong mechanism. The unusual kinetic behaviour when 2-hydroxy-5-nitrophenyl sulphate is the substrate is also consistent with this mechanism.

The formation of sulphate esters by the transfer of the sulphate group from adenosine 3'-phosphate 5'-sulphatophosphate to a suitable acceptor has been widely studied (see Roy & Trudinger, 1970). The reaction is catalysed by one of a group of enzymes, the sulphotransferases, each enzyme generally possessing a fairly narrow specificity with respect to the sulphate acceptor. In particular, a group of enzymes, phenol sulphotransferases (EC 2.8.2.1), catalysing transfer to a phenolic acceptor, have been isolated in mammalian tissues (Holczenberg & Rosen, 1965; Bostrom & Wengle, 1967). In some circumstances transfer of a sulphate group from *p*-nitrophenyl sulphate to a phenolic acceptor has been demonstrated (Gregory & Lipmann, 1957; Brunngraber, 1958). This crude phenol sulphotransferase from rabbit liver was shown to need adenosine 3',5'-bisphosphate as a cofactor and adenosine 3'-phosphate 5'-sulphatophosphate is presumably formed as an intermediate.

Burns & Wynn (1975) demonstrated that the rate of hydrolysis of a variety of phenolic sulphates by one of the arylsulphatases isolated from *Aspergillus oryzae* was greatly increased by the addition of suitable phenols. This increase was shown to be associated with an unusual phenol sulphotransferase activity in which the sulphate group is transferred directly from a phenolic sulphate such as 2-hydroxy-5-nitrophenyl sulphate to the free phenol without the intermediate formation of adenosine 3'-phosphate 5'-sulphatophosphate. In the present paper we report the results of kinetic studies with *p*-nitrophenyl sulphate as donor and tyramine as acceptor, and an explanation of the unusual kinetic behaviour observed when 2-hydroxy-5-nitrophenyl sulphate is the substrate.

Experimental

Materials

Preparation of dipotassium 4-nitrocatechol disulphate. Sulphation of 4-nitrocatechol (Ralph N. Emanuel, Wembley, Middx., U.K.) was carried out

as in the preparation of *p*-nitrophenyl sulphate by the method of Burkhardt & Lapworth (1926) as modified by Dodgson & Spencer (1953) except that a 3-fold molar excess of chlorosulphonic acid was used. The resulting thick green syrup was poured slowly with stirring into 30 ml of water containing 17 g of KOH, care being taken to ensure that the temperature did not rise above 35°C. Diethylaniline was removed by four successive extractions with 100 ml portions of diethyl ether. The aqueous layer was acidified to pH 4 with dilute H₂SO₄ and extracted with a further 4 × 100 ml of diethyl ether to remove 4-nitrocatechol. The pH was adjusted to pH 9 with KOH and the insoluble material was collected by filtration and then dissolved in the minimum quantity of water. Ethanol was added slowly with shaking until a fine white precipitate formed. The precipitate (largely inorganic sulphate) was removed by filtration, and a 10-fold excess of ethanol was added to the filtrate. Diethyl ether was added to the mixture (1:5, v/v) and the resulting yellow precipitate was filtered and dried. This material was free from inorganic sulphate. The precipitate was resuspended in a small volume of ethanol and warmed on a steam bath. Water was added slowly so as just to effect solution, and the solution was left to cool at room temperature (20°C). The first crystals to form were white, but on further standing, yellow crystals began to form. At this point, the crystals were collected by filtration and the recrystallization was repeated twice, yielding about 0.3 g of white crystals. Analysis gave the following percentage composition: C, 18.3; H, 0.6; N, 3.5; S, 16.5% (calc. for dipotassium 4-nitrocatechol disulphate, C₆H₃NO₁₀K₂S₂: C, 18.4; H, 0.7; N, 3.6; S, 16.4%).

Other substrates and enzyme. The potassium salt of *p*-nitrophenyl sulphate was prepared by the method of Burkhardt & Lapworth (1926) as modified by Dodgson & Spencer (1953). Dipotassium 2-hydroxy-5-nitrophenyl sulphate was prepared by the method of Roy (1953) as modified by Dodgson & Spencer (1956). Tyramine hydrochloride was from Sigma

(London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Phenol sulphotransferase (arylsulphatase II) was purified from an acetone-dried powder of *A. oryzae* as described previously (Burns & Wynn, 1975).

Methods

Determination of arylsulphatase activity. Preliminary experiments indicated that the rate of hydrolysis of both substrates was a linear function of time over the range studied, so 0.5 ml of undiluted stock enzyme was incubated for 30 min at 37°C with 0.5 ml of substrate solution in the range 0–10 mM. Reactions were terminated by the addition of 2 ml of 0.5 M-NaOH. Preliminary experiments had shown that the pH optimum of the arylsulphatase and phenol sulphotransferase activities were pH 7.6, so 0.2 M-Tris/HCl buffer, pH 7.6, was used in all kinetic experiments.

Kinetic assay of phenolsulphotransferase activity. Substrate solutions were prepared in 0.2 M-Tris/HCl buffer, pH 7.6. Preliminary experiments showed that the rate of reaction was a linear function of enzyme concentration, so the stock enzyme solution was diluted to give suitable rates of reaction over the range of substrate concentrations used. Both substrate and enzyme solutions were preincubated at 25°C, and 2 ml of enzyme solution was added to 2 ml of substrate solution. Then 0.5 ml samples were removed at 2 min intervals, added to 2.5 ml of 0.5 M-NaOH and the A_{402} was measured against an appropriate blank in which buffer replaced enzyme solution. The resulting absorbances were plotted against time and yielded linear plots. The initial rates were calculated from the gradients of these plots, which were computed by using the method of least squares. Kinetic parameters were computed by the method of Wilkinson (1961).

Thin-layer chromatography. Undiluted enzyme (0.5 ml) was added to 0.5 ml of 15 mM-2-hydroxy-5-nitrophenyl sulphate dissolved in 0.2 M-Tris/HCl, pH 7.6, and incubated for 6 h at 37°C. A control was included in which buffer replaced enzyme solution. Portions (5 μ l) were then applied as spots on silica-gel F₂₅₄ precoated plates (5 cm $\times$ 10 cm; layer thickness 0.25 mm: E. Merck, Darmstadt, Germany). Authentic standards were spotted alongside the incubation mixtures. Two solvent systems were used: I, butan-1-ol/acetic acid/water (50:17:25, by vol.); II, ethyl acetate/acetone/acetic acid/water (10:4:2:1, by vol.). The plates were then developed and left to dry, and components were detected either directly after exposure to NH₃ or under u.v. light (254 nm), when they appeared as pink spots.

In a further experiment, 50 μ l portions of the enzyme incubation mixture were applied as a streak to thin-layer plates and the chromatograms developed in solvent system I. The gel containing the unknown

component was scraped off the plates and the material eluted with water. A small portion of the resulting solution was re-applied as a spot to another thin-layer plate, and an equal volume of 2 M-HCl was added to the remaining solution, which was then heated for 1 h at 100°C. After cooling, the solution was extracted with a small quantity of diethyl ether and 10 μ l of the ether extract was spotted alongside the unhydrolysed sample. The ether was evaporated from the remaining solution and the residue dissolved in 0.5 M-NaOH. The absorbance spectrum of this solution was recorded and compared with that obtained from a sample of 4-nitrocatechol. The plates were developed in solvent system I and components located as described above.

Results

Comparison of the hydrolysis of p-nitrophenyl sulphate and 2-hydroxy-5-nitrophenyl sulphate in the absence of added phenols

Double-reciprocal plots of the variation of activity with substrate concentration are shown in Fig. 1. At low substrate concentrations, the data for 2-hydroxy-5-nitrophenyl sulphate yielded curved plots. At higher concentrations, the plots become linear. When *p*-nitrophenyl sulphate was the substrate, linear plots were observed over all concentration ranges. When the linear plots were extrapolated, V appeared to be greater for 2-hydroxy-5-nitrophenyl sulphate than for *p*-nitrophenyl sulphate.

Hydrolysis of p-nitrophenyl sulphate in the presence of tyramine

The effect of varying the concentration of *p*-nitrophenyl sulphate on the rate of formation of *p*-nitrophenol at several fixed concentrations of tyramine is shown in Fig. 2. Double-reciprocal plots produced a series of parallel lines, i.e. the apparent K_m for *p*-nitrophenyl sulphate divided by V was a constant of average value 5.34 and falling in the range 5.34 ± 0.55 regardless of the concentration of the fixed substrate. The data is thus consistent with the transferase reaction proceeding by a Ping Pong mechanism.

Similar plots of the effect of varying concentrations of tyramine on the rate of formation of *p*-nitrophenol at several concentrations of *p*-nitrophenyl sulphate were also parallel (average $K_m/V = 2.18$ falling in the range 2.18 ± 0.11) consistent with a Ping Pong mechanism.

Secondary plots of the data, where the reciprocals of the apparent K_m and V for tyramine were plotted against the reciprocal of the *p*-nitrophenyl sulphate concentration, gave a K_m for tyramine at infinite concentration of *p*-nitrophenyl sulphate of 2.0 mM,

Fig. 1. Double-reciprocal plot of rate versus substrate concentration in the absence of tyramine

Reactions were carried out at 37°C in 0.2M-Tris/HCl buffer, pH 7.6; 0.5ml of stock enzyme solution was added to 0.5ml of substrate solution and the reaction was stopped after 30min by the addition of 2ml of 0.5M-NaOH. Substrates were 2-hydroxy-5-nitrophenyl sulphate (●) and *p*-nitrophenyl sulphate (▲).

and a K_m for *p*-nitrophenyl sulphate at infinite concentration of tyramine of 4.3 mM. V in both cases was determined to be 0.87 $\mu\text{mol/min per ml}$ of enzyme solution.

Identification of 4-nitrocatechol disulphate in enzyme reaction mixtures containing 2-hydroxy-5-nitrophenyl sulphate as the sole substrate

When enzyme incubation mixtures containing 2-hydroxy-5-nitrophenyl sulphate as the sole substrate were subjected to t.l.c., three components could be identified. The R_F values of these components are shown in Table 1 together with the R_F values of 2-hydroxy-5-nitrophenyl sulphate, 4-nitrocatechol and 4-nitrocatechol disulphate. From these results the unknown component appeared to be 4-nitrocatechol disulphate. Acid hydrolysis of this component followed by further chromatographic analysis revealed the presence of a single component having identical chromatographic behaviour with 4-nitrocatechol and an identical absorption spectrum, con-

Fig. 2. Double-reciprocal plots of rate versus substrate concentration with *p*-nitrophenyl sulphate as the variable substrate

Reactions were carried out at 25°C in 0.2M-Tris/HCl buffer, pH 7.6. Tyramine was present at concentrations of 0.1 M (●), 0.2 mM (○), 0.4 mM (▲), 0.6 mM (□) and 0.8 mM (■). For further details see the text.

firming that the component was indeed 4-nitrocatechol disulphate.

Discussion

The demonstration of a Ping Pong mechanism for the transfer reaction, together with the observation that free sulphate is not incorporated into the product (Burns & Wynn, 1975), provides strong evidence that the reaction proceeds via the formation of a covalent enzyme-sulphate intermediate. The arylsulphatase activity of this enzyme, where water substitutes for the phenolic acceptor, presumably

Table 1. *Thin-layer chromatography of reaction mixtures incubated with 2-hydroxy-5-nitrophenyl sulphate alone*
Enzyme and substrate were incubated together as described in the Experimental section. Spots (5 μ l) of the mixtures and of standard solution were then applied to silica-gel plates and the chromatograms developed in solvent systems I and II.

Solution	R_F	
	Solvent system I	Solvent system II
Enzyme incubation mixture	0.27, 0.63, 0.82	0.29, 0.69, 0.97
4-Nitrocatechol disulphate	0.27	0.29
2-Hydroxy-5-nitrophenyl sulphate	0.63	0.69
4-Nitrocatechol	0.82	0.97

proceeds by the same mechanism, with the breakdown of the enzyme-sulphate intermediate as the rate-limiting step. This proposed mechanism requires that the hydrolytic activity of the enzyme exhibits a maximum velocity independent of the substrate used. The much greater maximum velocity observed with 2-hydroxy-5-nitrophenyl sulphate than with *p*-nitrophenyl sulphate appears to be inconsistent with this requirement. However, the observation that 4-nitrocatechol disulphate is produced when the former substrate is used indicates that the reaction is not purely hydrolytic and that the free hydroxyl group confers acceptor properties on this substrate, so that the reaction may be formulated as in Scheme 1.

The right-hand loop of Scheme 1 represents the purely hydrolytic activity and the outer rim represents the purely sulphotransferase activity. Assuming initial-rate conditions and that rate constants involving the formation or breakage of covalent bonds are small compared with those involving simple association, steady-state treatment yields the following rate equation:

$$v = \frac{(k_{+1}k_{+2}k_{-4}k_{+7}/k_{+1}k_{+2}k_{-4} + k_{-1}k_{+4}k_{+5})[S] + (k_{+1}k_{+2}k_{+4}k_{+5}/k_{+1}k_{+2}k_{-4} + k_{-1}k_{+4}k_{+5})[S]^2}{(k_{-1}k_{-4}k_{+7}/k_{+1}k_{+2}k_{-4} + k_{-1}k_{+4}k_{+5}) + [S] + [k_{+1}k_{+4}(k_{+2} + k_{+5})/(k_{+1}k_{+2}k_{-4} + k_{-1}k_{+4}k_{+5})][S]^2}$$

or

$$v = \frac{a[S] + b[S]^2}{c + [S] + d[S]^2}$$

since a and c involve terms in k_{+7} they can be ignored except at very low concentrations of $[S]$. At high concentrations of $[S]$ the equation simplifies to:

$$v = \frac{\frac{b}{d} \cdot [S]}{\frac{1}{d} + [S]}$$

Scheme 1. *Schematic representation of the formation by arylsulphatase II of 4-nitrocatechol disulphate from 2-hydroxy-5-nitrophenyl sulphate*

E, free uncomplexed enzyme; E', enzyme-sulphate intermediate.

i.e. straight-line double-reciprocal plots will result. At low substrate concentrations the equation cannot be simplified and curved plots are predicted. The data of Fig. 1 is thus consistent with the proposed Ping Pong mechanism.

It is pertinent to note that Mayers & Kaiser (1968) have suggested that a compound related to the sulphur trioxide adduct of imidazole might be involved in the sulphotransferase reaction. The pH optimum of the reaction is consistent with the participation of histidine residues in the catalysis. Benkovic (1971) has considered a number of model reactions for the catalysis of phosphate and sulphate transfer, including a number which involve imidazole groups, and Kiefer *et al.* (1972) produced a synthetic enzyme based on polyethyleneimine which contained only

dodecyl side chains for binding substrate and imidazole moieties as functional catalytic groups. This polymer had a similar catalytic capability to the arylsulphatases with respect to the hydrolysis of 2-hydroxy-5-nitrocatechol sulphate, and moreover exhibited saturation kinetics. The reaction was observed to produce an initial burst of 4-nitrocatechol, which would be consistent with the reaction proceeding through the formation of a sulphated polymer intermediate, with subsequent breakdown as the rate-limiting step.

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