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KINETIC PROPERTIES OF THE SOLUBLE ADENOSINE 5′-PHOSPHOSULPHATE SULPHOHYDROLASE FROM BOVINE LIVER

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SUMMARY

The kinetic properties of the soluble adenosine 5′-phosphosulphate sulphohydrolase (APS sulphohydrolase) of bovine liver have been studied. The enzyme exhibits a sharply defined pH optimum (5.4) and is optimally active towards 4.0 mM APS above which concentration inhibition by excess substrate is observed.

AMP and inorganic sulphate, the products of the enzymic hydrolysis of APS, inhibit the enzyme non-competitively and competitively, respectively. This pattern of product inhibition is consistent with an ordered uni–bi reaction sequence with SO_4^{2-} as the last-released product. The formation of an intermediate enzyme–SO_4^{2-} complex is implicit in this mechanism.

The enzyme is inactivated (irreversibly inhibited) by incubation with ethoxyformic anhydride and by photo-oxidation in the presence of Rose Bengal. The rate of inactivation observed with these reagents is reduced in the presence of ATP, a known competitive inhibitor of APS sulphohydrolase. These results are consistent with the presence of an essential histidine residue at the active site of the enzyme.

A possible mechanism of action for APS sulphohydrolase is discussed in the light of these findings.

INTRODUCTION

Enzymes capable of degrading the so called active forms of sulphate, APS (adenosine 5′-phosphosulphate) and PAPS (3′-phosphoadenosine 5′-phosphosulphate) are widely distributed in the animal kingdom[1] (*e.g.* PAPS sulphohydrolase[2–5], PAPS 3′-nucleotidase and APS sulphohydrolase[1,6–8]). Apart from their possible physiological significance, a knowledge of these enzymes is of practical importance because, if due

Abbreviations: APS, adenosine 5′-phosphosulphate; PAPS, 3′-phosphoadenosine 5′-phosphosulphate.

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care is not observed, they may constitute undesirable components in experimental systems designed for the study of biological sulphation processes.

In previous communications from these laboratories[9,10] the separation of three PAPS-degrading enzymes from bovine liver cytosol was reported. The isolation of one of these (APS sulphohydrolase) in a homogeneous form has also been achieved[11]. The present paper describes some kinetic properties of APS sulphohydrolase and discusses the possible mechanism of action of the enzyme.

MATERIALS AND METHODS

Chemicals

APS and [^{35}S]APS were prepared by established procedures[11]. All other nucleotides were obtained from Sigma Chemical Co. Ltd, St. Louis, Mo., U.S.A. and were of the highest purity available. Rose Bengal and ethoxyformic anhydride were obtained from Eastman Organic Chemicals, Rochester 3, New York, N.Y., U.S.A.

Enzyme preparation

APS sulphohydrolase was prepared from fresh bovine liver according to the procedure of Stokes *et al.*[11] and migrated as a single enzymically active band on polyacrylamide disc gel electrophoresis in the pH range 7.2–8.9.

Assay conditions for kinetic studies

In the absence of a suitable continuous assay procedure, APS sulphohydrolase activity was determined by a method based on that of Bailey-Wood *et al.*[1] which involves the estimation of inorganic sulphate liberated from APS.

Unless otherwise stated, APS sulphohydrolase (protein concentration 0.01 mg/ml) was incubated at 30 °C with concentrations of [^{35}S]APS ranging from 0.1 to 2.0 mM in 0.2 M sodium acetate–acetic acid buffer, pH 5.4, in a total volume of 0.2 ml. After the removal of residual [^{35}S]APS[1] the liberated $^{35}SO_4^{2-}$ was determined by liquid scintillation spectrophotometry[11]. Throughout the following kinetic studies the minimum practical incubation period was employed (usually 2–4 min). Thus, less than 5% of the APS present was hydrolysed and no correction was made for this slight change in substrate concentration in subsequent calculations.

EXPERIMENTAL AND RESULTS

Effect of substrate concentration

Fig. 1 shows the effect of varying substrate concentration on the initial rate. Optimal enzymic activity was obtained with a substrate concentration of 4.0 mM. At APS concentrations greater than this, inhibition by excess substrate was observed. A Michaelis constant (K_m) of $9.5 \cdot 10^{-4}$ M was obtained from this data[12]; this value is similar to the K_m for rat liver supernatant APS sulphohydrolase quoted by Bailey-Wood *et al.*[1] as $1.0 \cdot 10^{-3}$ M.

Effect of pH on enzyme activity

The variation of initial reaction rate with substrate concentration was studied at different pH values in both sodium acetate–acetic acid buffer (0.2 M, pH 4.0–6.3)

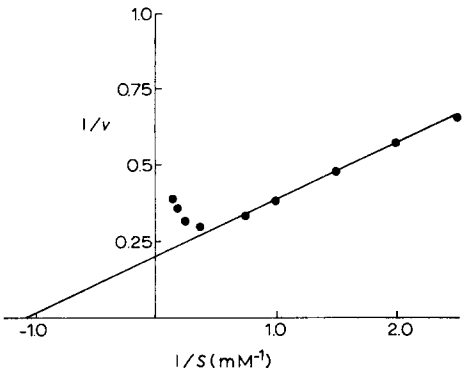

Fig. 1. The effect of substrate concentration *(S)* on the initial reaction rate *(v)*. Plot of $1/v$ against $1/S$ showing substrate inhibition.

and sodium citrate–citric acid buffer (0.125 M, pH 3.5–6.6). Liberated $^{35}SO_4^{2-}$ was assayed and the initial rate was calculated in each case. Maximum initial rates and K_m values were determined from double reciprocal plots[12] and the catalytic centre activity (k_{cat}, s^{-1}) of APS sulphohydrolase at each pH value was obtained from the maximum initial rate, assuming one catalytic centre per molecule of enzyme (based on a mol. wt of 68 000)[11]. The variation of k_{cat} with pH in acetate buffer is shown in Fig. 2. Similar results were obtained when citrate buffer was employed.

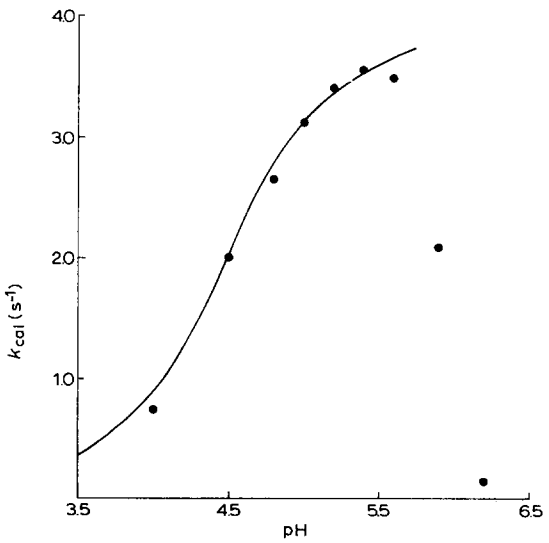

Fig. 2. The effect of pH on the catalytic centre activity (k_{cat}, s^{-1}) of APS sulphohydrolase in 0.2 M acetate buffer. The points represent experimental values, the curve describes a theoretical ionization with a pH value of 4.5.

Effect of pH on the stability of APS sulphohydrolase

APS sulphohydrolase (protein concentration 0.01 mg/ml) was incubated at 30 °C and 37 °C with either 0.2 M sodium acetate–acetic acid buffer (pH 5.2–5.8) or

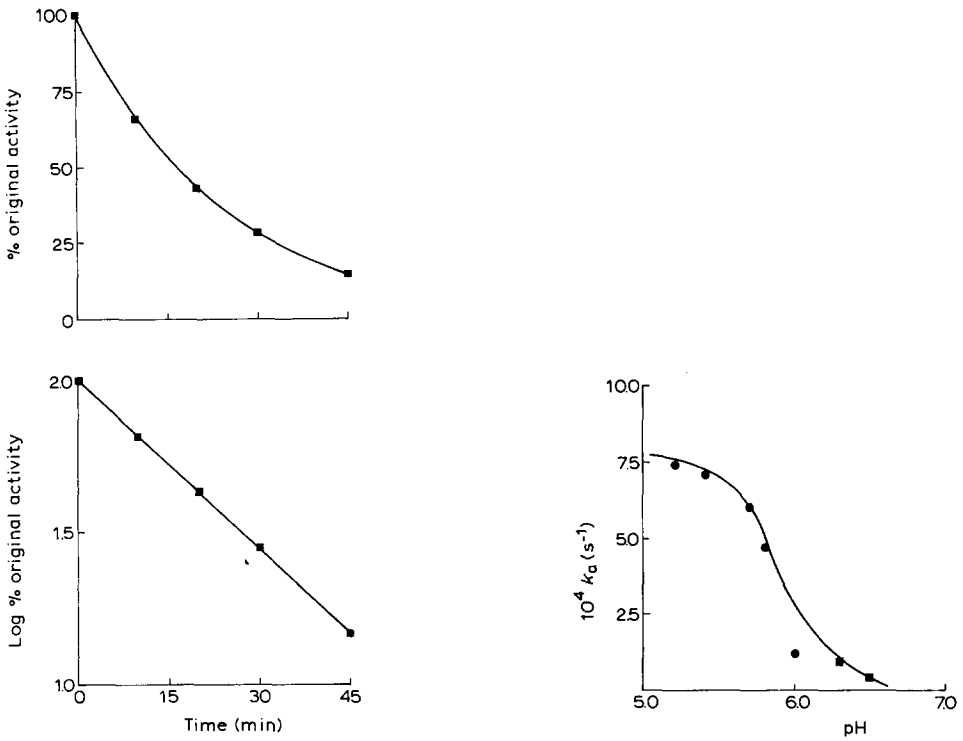

Fig. 3. Normal and semi-log progress curves for the loss of APS sulphohydrolase activity on preincubation at pH 5.4 and 37 °C. Activity is expressed as a percentage of that present before preincubation. For clarity, only this one example of the series of curves obtained at different pH values is included.

Fig. 4. The effect of pH on the apparent first-order rate constant (k_a, s^{-1}) for loss of APS sulphohydrolase activity in acetate buffer (●) and citrate buffer (■). The points are experimentally determined values, the curve describes a theoretical multiple ionization with a pK value of 5.8. Experimental details are given in the text.

0.125 M sodium citrate–citric acid buffer (pH 6.0–6.6). Samples were withdrawn after time periods of 15–45 min and assayed for enzyme activity in the usual way. A typical progress curve for the loss of activity observed at 37 °C is shown in Fig. 3. The variation with pH of the apparent first-order rate constant for loss of activity (determined from semi-logarithmic progress curves) is shown in Fig. 4. The experimental points can be seen to fit a theoretical curve which represents a model system involving multiple ionisations. This was calculated assuming the involvement of three such groups, each with a pK of 5.8, using the appropriate expression for the apparent first-order rate constant (k) for enzyme denaturation, measured at any pH, *viz.*

$$k = \bar{k} \Big/ \left[1 + \frac{K}{[H^+]} + \frac{K^2}{[H^+]^2} + \frac{K^3}{[H^+]^3}\right]$$

where $\bar{k}$ is the pH-independent first-order rate constant and K is the macroscopic ionisation constant for the ionising groups.

The pH-dependent inactivation was also found to be very temperature sensitive. At 30 °C less than 10% of the original activity was lost in 45 min at pH 5.4. Subsequent kinetic experiments were therefore performed under these conditions.

Effect of some metal ions on APS sulphohydrolase

Before testing the effect of metal ions the enzyme was exhaustively dialysed against 0.01 M EDTA in 0.02 M Tris–HCl buffer at pH 7.2, followed by dialysis against buffer alone. This treatment had no appreciable effect on enzyme activity.

Enzyme protein (0.01 mg) was preincubated at 30 °C with metal ion at a final concentration of 0.2 mM (0.4 mM in the case of K^+) at either pH 5.2 in 0.1 M sodium acetate–acetic acid buffer or at pH 7.2 in 0.1 M Tris–HCl. With the exception of lead acetate, the metals were present as chloride salts. All controls contained 0.4 mM NaCl. Samples were withdrawn after 5 min preincubation and then assayed for enzyme activity in the usual way. The enzyme showed no requirements for metal ions and the results obtained (Table I) are very similar to those noted by Bailey-Wood *et al.*[1] for the analogous rat liver cytosol enzyme.

TABLE I

EFFECT OF SOME METAL IONS ON APS SULPHOHYDROLASE

Enzyme and metal ions were preincubated for 5 min as described in the text before assaying in the usual manner.

Metal ion	*Inhibition (%)*	
	pH 5.2	*pH 7.2*
Mg^{2+}	5	8
Ca^{2+}	6	4
Mn^{2+}	5	5
Fe^{2+}	11	9
Co^{2+}	1	3
Ni^{2+}	6	8
Cu^{2+}	93	86
Hg^{2+}	98	95
Pb^{2+}	4	10
K^+	2	1

Product inhibition of APS sulphohydrolase

The variation of initial rate with substrate concentration was studied in the presence of SO_4^{2-} at concentrations of up to 25 mM. Similar experiments were performed in which the SO_4^{2-} was replaced by AMP (at concentrations up to 4.0 mM). Controls contained neither AMP nor SO_4^{2-}. Results are present as double reciprocal plots[12] in Fig. 5.

AMP exhibited typical noncompetitive (mixed) kinetics (K_i $1.5 \cdot 10^{-3}$ M) whereas SO_4^{2-} exhibited purely competitive kinetics (K_i $1.5 \cdot 10^{-2}$ M). This pattern of product inhibition is consistent with an ordered uni–bi reaction sequence with SO_4^{2-} as the last-released product

$$E + \text{APS} \rightleftharpoons E\text{–APS} \rightleftharpoons E\text{–}SO_4^{2-} + \text{AMP} \rightleftharpoons E + SO_4^{2-}$$

Possible unimolecular transitions (kinetically indistinguishable) are omitted

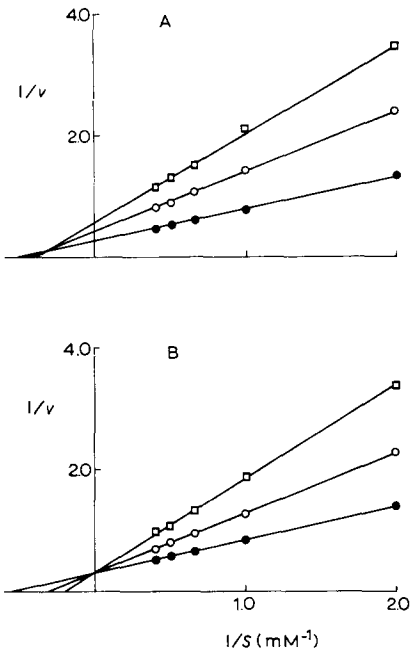

Fig. 5. Product inhibition of APS sulphohydrolase. The effect of AMP and SO_4^{2-} on the initial rate *(v)* in the presence of different concentrations of APS *(S)*. Plots of $1/v$ against $1/S$ showing non-competitive (mixed) and competitive inhibition, respectively. (A) ●, no inhibitor; ○, $2.0 \cdot 10^{-3}$ M AMP; □, $4.0 \cdot 10^{-3}$ M AMP. (B) ●, no inhibitor; ○, $1.25 \cdot 10^{-2}$ M SO_4^{2-}; □, $2.50 \cdot 10^{-2}$ M SO_4^{2-}.

from this scheme. It is of some interest that the formation of an intermediate enzyme–SO_4^{2-} complex is implicit in this mechanism.

Inhibition by nucleotides

The effect of the following nucleotides (in the concentration ranges shown) on the variation of initial rate with substrate concentration was determined: ATP ($2.5 \cdot 10^{-5}$–$5.0 \cdot 10^{-5}$ M), GTP ($2.0 \cdot 10^{-5}$–$4.0 \cdot 10^{-5}$ M), ITP ($1.0 \cdot 10^{-4}$–$2.0 \cdot 10^{-4}$ M), UTP (2.5–$5.0 \cdot 10^{-4}$ M), adenosine 3′-monophosphate (2.04–$4.0 \cdot 10^{-3}$ M), adenosine 2′-monophosphate (2.0–$4.0 \cdot 10^{-3}$ M), cyclic AMP (2.0–$4.0 \cdot 10^{-3}$ M), ADP (2.5–$5.0 \cdot 10^{-4}$ M) and adenosine (0.5–$1.0 \cdot 10^{-2}$ M). Control incubations were performed in the absence of inhibitor. The type of inhibition occurring in each instance was determined from a double reciprocal plot[12] and the inhibitor constant obtained from a plot of reciprocal initial rate against inhibitor concentration[13]. The results are summarized in Table II.

Effect of some group-specific reagents on APS sulphohydrolase

APS sulphohydrolase (protein concentration 0.01 mg/ml) was incubated for periods of up to 90 min at 30 °C with varying concentrations of reagents in 0.2 M sodium acetate–acetic acid buffer, pH 5.4. When ethoxyformic anhydride was present 0.1 M acetate buffer, pH 6.1, was employed[14]. Control incubations were performed under identical conditions but in the absence of reagents. Samples were withdrawn after 5, 30 and 90 min and assayed for APS sulphohydrolase activity in the usual

TABLE II

INHIBITION OF APS SULPHOHYDROLASE BY NUCLEOTIDES

Inhibitor	*Inhibitor type*	*Inhibitor constant (K_i,M)*
ATP	Competitive	$7.5 \cdot 10^{-6}$
GTP	Competitive	$9.0 \cdot 10^{-6}$
ITP	Competitive	$3.4 \cdot 10^{-5}$
UTP	Competitive	$1.5 \cdot 10^{-4}$
Cyclic AMP	Competitive	$9.7 \cdot 10^{-3}$
Adenosine 3′-monophosphate	Competitive	$2.0 \cdot 10^{-3}$
Adenosine 2′-monophosphate	Competitive	$4.8 \cdot 10^{-3}$
ADP	Noncompetitive	$2.5 \cdot 10^{-5}$
AMP	Noncompetitive	$1.5 \cdot 10^{-3}$
Adenosine	Noncompetitive	$5.0 \cdot 10^{-3}$

way. The following reagents were tested in the concentration ranges shown: *p*-chloromercuribenzoic acid, 0.05–5.0 mM; 5′,5′-dithiobis-(2-nitrobenzoate), 0.05–5.0 mM; hydroxylamine, 0.05–6.25 mM and diisopropylfluorophosphate, 5.0 mM.

Most of these reagents had little effect on the enzyme. However, in the case of ethoxyformic anhydride (6.25 mM) 77% inhibition occurred in 5 min and inhibition was complete after 30 min. Attempts to reverse this inhibition by dialysis were unsuccessful indicating that the inhibition of APS sulphohydrolase by ethoxyformic anhydride is essentially irreversible and that covalent modification of the enzyme occurs.

The effect of ethoxyformic anhydride concentration (0.625–6.25 mM) on the rate of inactivation of APS sulphohydrolase was examined in the presence and absence of 0.1 mM ATP, a known competitive inhibitor of the enzyme. Considerable protection was afforded in the presence of the competitive inhibitor (Fig. 6A) but otherwise inactivation was extremely rapid and followed first-order kinetics except for an initial deviation (Fig. 6B). This effect was observed in all experiments.

The inactivation of APS sulphohydrolase by ethoxyformic anhydride implies the presence of an essential histidine residue at the active site of the enzyme. Attempts were made to follow the reaction spectrophotometrically by measuring the increase in absorbance at 242 nm due to the formation of ethoxyformylhistidine residues[15]. An increase in absorbance at this wavelength accompanied inactivation but accurate quantitation was not possible.

Photochemical inactivation of APS sulphohydrolase

The photoactive dye Rose Bengal[16] was employed in an attempt to confirm the presence of an essential histidine residue at the active site of APS sulphohydrolase.

Rose Bengal (4 μg) and APS sulphohydrolase (0.5 mg) were dissolved in 0.1 M Tris–HCl buffer, pH 7.2, contained in a quartz cell of 1.0 cm pathlength (total volume 1 ml). The system was irradiated by a focussed beam from a 500 W lamp placed 10 cm from the cell and maintained at 30 °C by means of a stream of cool air. Portions were removed at time intervals and assayed for APS sulphohydrolase activity in the usual way. Similar experiments were performed in the presence of 0.5 mM ATP. In control

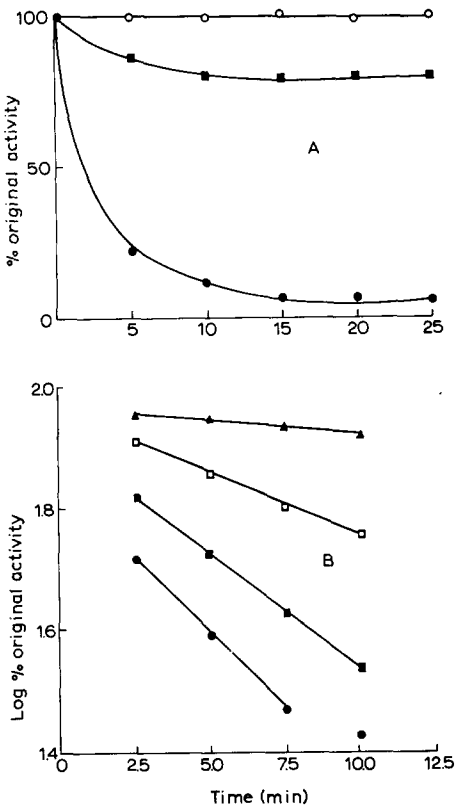

Fig. 6. (A) Typical progress curves for the inactivation of APS sulphohydrolase by ethoxyformic anhydride. ●, 6.25 mM ethoxyformic anhydride; ■, 6.25 mM ethoxyformic anhydride and 0.1 mM ATP; ○, control. (B) Semi-logarithmic progress curves for the inactivation of APS sulphohydrolase by different concentrations of ethoxyformic anhydride. ●, 4.25 mM; ■, 2.50 mM; □, 1.25 mM; ▲, 0.625 mM.

experiments either the whole system was kept in the dark or Rose Bengal was omitted.

Considerable inactivation of the enzyme occurred and ATP offered an appreciable degree of protection (Fig. 7A), indicating an event at the active site. The photoinactivation followed first-order kinetics apart from an initial deviation (Fig. 7B).

DISCUSSION

The present work established that the APS sulphohydrolase of bovine liver cytosol is kinetically very similar to the corresponding rat liver enzyme[1] but does differ in one important respect. The enzymes display similar K_m values but substrate inhibition is observed only with the bovine liver enzyme. The APS sulphohydrolase from pig kidney[17], in common with the rat liver enzyme, is not inhibited by excess substrate but exhibits a much lower K_m value than either of the liver enzymes and is also quite different in its response to changes of pH. The apparent differences in

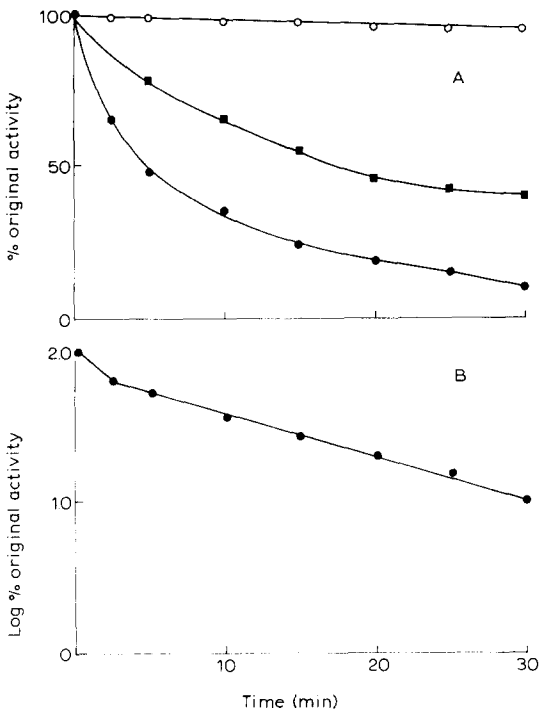

Fig. 7. (A) Typical progress curves for the photo-oxidation of APS sulphohydrolase in the presence of Rose Bengal. ●, Rose Bengal; ■, Rose Bengal and 0.5 mM ATP; ○, control. (B) Semi-logarithmic progress curve for the photo-oxidation of APS sulphohydrolase in the presence of Rose Bengal.

the properties of these enzymes may be resolved when studies with purified preparations are undertaken.

The absence of a distinct plateau of enzyme activity at the pH optimum of APS sulphohydrolase precludes the use of logarithmic activity–pH plots to determine pK values. However, a pK value of 4.5 has been obtained for the acid limb of the pH–activity plot by theoretical curve fitting. This value is consistent with the participation of a carboxyl group in the action of the enzyme. Similar pH–activity relationships have been observed with other sulphohydrolases (*e.g.* rat liver APS sulphohydrolase[1] and arylsulphohydrolase A[18]) but attempts to confirm the possible involvement of a carboxyl group in the action of arylsulphohydrolase A[18] have proved unsuccessful.

It is of some interest that an intermediate enzyme–SO_4^{2-} complex is implicit in the reaction sequence of APS sulphohydrolase indicated by the observed pattern of product inhibition. The chemical nature of such an intermediate cannot, however, be deduced directly from kinetic data. Attempts to demonstrate the existence of an enzyme–SO_4^{2-} complex by isolating the enzyme protein labelled with $^{35}SO_4^{2-}$ have so far proved unsuccessful. A similar ordered uni–bi reaction sequence (with SO_4^{2-} as the obligatory product) has been proposed for arylsulphohydrolase A[18] on the basis of competitive inhibition by SO_4^{2-}.

The origin of noncompetitive nucleotide inhibition of APS sulphohydrolase

(assuming one nucleotide binding site per molecule of enzyme) presumably lies in the combination of the inhibitor with the enzyme–SO_4^{2-} complex (*e.g.* AMP). Combination of APS with this complex could give rise to a nonproductive or dead-end complex and account for the substrate inhibition observed with APS sulphohydrolase. Nucleotides which are purely competitive inhibitors of the enzyme are presumably unable to combine with the intermediate enzyme–SO_4^{2-} complex, either because of molecular size (*e.g.* ATP) or steric restrictions (*e.g.* adenosine 3'-monophosphate).

There is a further apparent similarity between APS sulphohydrolase and arylsulphohydrolase A in the suggested involvement of histidine residues, directly or indirectly, in the mechanism of both enzymes. Benkovic and Dunikoski[19] have shown that the hydrolysis of 2,5(5)-imidazolylphenyl sulphate proceeds by an intramolecular catalysis, probably through the imidazole moiety acting as a general acid–base catalyst. Furthermore it has recently been demonstrated that a synthetic polymer (polyethyleneimine) containing only histidine residues as functional groups, is capable of catalysing a 10^{12}-fold rate acceleration of the hydrolysis of nitrocatechol sulphate (a substrate for arylsulphohydrolase A)[20]. It seems possible that general acid–base catalysis by imidazole might represent a general feature of at least some sulphohydrolases.

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