The effect of pH on the kinetics of arylsulphatases A and B

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The effect of pH on the kinetics of rat liver arylsulphatases A and B is very similar and shows that two groups with pK values of 4.4–4.5 and 5.7–5.8 are important for enzyme activity. Substrate binding has no effect on the group with a pK of 4.4–4.5; however, the pK of the second group is shifted to 7.1–7.5 in the enzyme–substrate complex. An analysis of the effect of pH on the $K_v$ for sulphate inhibition suggests that $HSO_4^-$ is the true product. A model is proposed that involves the two ionizing groups identified in the present study in a concerted general acid–base-catalysed mechanism.

Several sulphatases have been described that catalyse the desulphation of a variety of sugar sulphate substrates. The existence of many of these enzymes was inferred from clinical syndromes where increased excretion and/or accumulation of sulphated sugar derivatives was evident (see, e.g., McKusick et al., 1978, for review).

Recently, several of these enzymes, e.g. N-acetylgalactosamine 6-sulphate sulphatase (Basner et al., 1979), N-acetylgalactosamine 6-sulphate sulphohydrolase (Lim & Horwitz, 1981) and glucosamine ON-disulphate O-sulphohydrolase (Weissman et al., 1980) have been purified. Two lysosomal enzymes, arylsulphatases A and B, have been known for many years, since their activity is easily measured by using 2-hydroxy-5-nitrophenyl sulphate as substrate. Arylsulphatase A has recently been shown to catalyse the desulphation of lipid-linked galactose 3-sulphate (Mehl & Jatzkewitz, 1968; Yamato et al., 1974; Fluharty et al., 1974), whereas arylsulphatase B catalyses the desulphation of N-acetylgalactosamine 4-sulphate (O’Brien et al., 1974; Fluharty et al., 1975). None of the sulphatases are particularly abundant proteins and the assays using sugar sulphates as substrates are time-consuming, so that few mechanistic studies have been reported. We have analysed the pH-dependence of arylsulphatases A and B with 2-hydroxy-5-nitrophenyl sulphate in an attempt to identify functionally important ionizing groups in these two proteins.

Partial purification of rat liver arylsulphatases A and B

Rat liver arylsulphatases A and B were partially purified as previously described (O’Fagain et al., 1982).

Assay of arylsulphatase activity

Arylsulphatase activity was measured by monitoring the production of 4-nitrocatechol and 4-nitropheno1 from 2-hydroxy-5-nitrophenyl sulphate and 4-nitropheno1 sulphate respectively. Assays were conducted at 30°C in a final volume of 0.5 ml containing buffer 0.33 M-sodium acetate/acetic acid, pH 4–6; 0.33 M-Mes (4-morpholine-ethanesulfonic acid)/NaOH, pH 5.6–7.1; 0.2 M-Tris/acetate, pH 6.2–8.0) and substrate dissolved in the appropriate buffer (and titrated if necessary). The reaction was normally started by the addition of 10 μl of the enzyme and terminated by the addition of 1 M-NaOH. The 4-nitrocatechol produced was measured by its $A_{515}$ ($ε = 12600$ litre·mol$^{-1}$·cm$^{-1}$; Waheed & Van Etten, 1979) and the 4-nitropheno1 by the $A_{400}$ ($ε = 18200$ litre·mol$^{-1}$·cm$^{-1}$; Dodgson & Spencer, 1953).

Treatment of data

All initial rate data were plotted in double-reciprocal form. The slopes $(K_m/V')$ and intercepts $(1/V)$ of each line were estimated by fitting the data to eqn. (1) by the method of Wilkinson (1961):

$$v = \frac{V[S]}{K_m + [S]} \tag{1}$$

When $Na_2SO_4$ was used as a reversible inhibitor, the slope replots were fitted to a straight line by linear regression and the $K_i$ was obtained by extrapolating...
to the inhibitor-concentration axis. The $V$ and $V/K_m$ values obtained at different pH values were fitted by a least-squares procedure to eqn. (2):

$$f = \frac{\hat{f}}{1 + \frac{H}{K_2} + \frac{H}{K_1}}$$

where $f$ is the measured kinetic parameter, $\hat{f}$ is the 'pH-corrected' parameter, $H$ is the hydrogen-ion concentration and $K_1$ and $K_2$ are dissociation constants.

**Results**

The pH-stability of both enzymes was demonstrated by pre-incubating them for various periods at the extremes of pH used in the kinetic experiments before assaying activity at pH 5.9. In these and subsequent experiments, initial rates were measured over a few minutes. This is particularly important for arylsulphatase A, which exhibits a slow reversible substrate-dependent inactivation (O’Fagain et al., 1982). As some of the data sets (e.g. the variation of the $K_1$ for sulphate with pH) took several days to complete, the pH examined on the first day was repeated on completion of the experiment. These experiments showed that $K_m$ values were unaffected. Decreases in $V$ not exceeding 10% were encountered with arylsulphatase B, whereas arylsulphatase A remained completely stable.

For both arylsulphatases A and B, two pK values of 4.4–4.5 and 7.1–7.5 are apparent in the $V$ data (Figs. 1a and 2a). Two pK’s are also seen in the $V/K_m$ data, yielding values of 4.4 and 5.8 for arylsulphatase A and 4.5 and 5.7 for arylsulphatase B (Figs. 1b and 2b). The pK of 7.5 in Fig. 1(a) is a least-squares estimate, although any pK above 7 would equally well fit the data shown in Figs. 1(a) and 1(c). Similarly, the pK of 7.1 used in the theoretical continuous curve for Fig. 2(c) is derived from the points in Fig. 2(a) and used in Fig. 2(c), although any pK above 7 would equally well fit the data of Fig. 2(c). It is only in Fig. 3(c) that the alkaline pK is readily apparent in the $K_m$ data. It should be noted that pK’s etc. are molecular pK values and not group pK values (see, e.g., Dixon, 1976). The results of similar experiments using 4-nitrophenyl sulphate as substrate for arylsulphatase B are shown in Fig. 3. The effect of pH on the $K_1$ for sulphate against arylsulphatase A and B is shown in Fig. 4. Despite a pronounced ‘buffer effect’, it is clear that lowering the pH enhances the inhibition by sulphate. For both enzymes the inhibition by sulphate remained strictly competitive over the entire range of pH studied. When a similar experiment was conducted with orthophosphate as a competitive inhibitor of arylsulphatase B (O’Fagain et al., 1980), the $K_1$ was independent of pH over the range 4.5–5.9. The $K_1$ values obtained were 35 μM, 24 μM and 47 μM at pH values of 4.5, 5.2 and 5.9 respectively.

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Fig. 1. Effect of pH on the kinetics of arylsulphatase A

The effect of pH on the kinetic parameters $V$ and $V/K_m$ using 2-hydroxy-5-nitrophenyl sulphate as substrate are shown in (a) and (b) respectively. Data points are shown for acetate (O) and Mes (●) buffers. The continuous lines are least-squares fits to eqn. (2), where $K_1$, $K_2$ and $f$ are, for (a), $4 \times 10^{-3}$M, $3 \times 10^{-8}$M and 6, and for (b), $4 \times 10^{-5}$M, $1.5 \times 10^{-6}$M and $1.45 \times 10^{-2}$ respectively. The effect of pH on $K_m$ is shown in (c) to point out that $K_m$ is not sensitive to the ionizations characterized by $K_1$ and $K_2$. The experiments illustrated in Figs. 1, 2 and 3 were conducted with 1mg of partially purified enzyme.
pH kinetics of arylsulphatases

Fig. 2. Effect of pH on the kinetics of arylsulphatase B

The effect of pH on the kinetic parameters \( V \) and \( V/K_m \) using 2-hydroxy-5-nitrophenyl sulphate as substrate are shown in (a) and (b) respectively. Data points are shown for acetate (O), Mes (●) and Tris (□) buffers. The continuous lines are least-squares fits to eqn. (2), where \( K_1 \), \( K_2 \) and \( f \) are, for (a), \( 3 \times 10^{-5}\)M, \( 8 \times 10^{-8}\)M and 7.5, and for (b), \( 3 \times 10^{-5}\)M, \( 2 \times 10^{-6}\)M and 1.15 \( \times 10^{-3} \) respectively. The effect of pH on \( K_m \) is shown in (c) to point out that \( K_m \) is not sensitive to the ionizations characterized by \( K_1 \) and \( K'_1 \).

Discussion

As both arylsulphatases A and B exhibit a similar pH-dependence of their kinetic parameters and as differences in the observed pK values for the two

Vol. 213

Fig. 3. pH kinetics of arylsulphatase B with 4-nitrophenyl sulphate as substrate

The effect of pH on the kinetic parameters \( V \) and \( V/K_m \) are shown in (a) and (b) respectively. Data points are shown for acetate (O) and Tris (●) buffers. The continuous lines are based on eqn. (2), where \( K_1 \), \( K_2 \) and \( f \) are, for (a), \( 10^{-3}\)M, \( 8 \times 10^{-8}\)M and 0.18, and for (b), \( 10^{-3}\)M, \( 5 \times 10^{-7}\)M and \( 4.5 \times 10^{-6} \) respectively. The effect of pH on \( K_m \) is shown in (c) to point out that \( K_m \) is not sensitive to the ionizations characterized by \( K_1 \) and \( K'_1 \).
enzymes are small, no distinction is made between them in the following discussion.

Our analysis of the pH effects is based on the model shown in Scheme 1. The initial-rate equation for Scheme 1, with the normal assumption of quasi-equilibrium (see e.g. Cornish-Bowden, 1976; Brocklehurst & Dixon, 1976), is:

\[ \nu = \frac{V[S]}{K_m\left(1 + \frac{H}{K_1} + \frac{K_2}{K'_1 + H}\right) + [S]\left(1 + \frac{H}{K'_1} + \frac{K'_2}{H}\right)} \]  

(3)

The two \( pK \)'s of the enzyme–substrate complex, \( K'_1 \) and \( K'_2 \) are apparent in the \( V \) data, whereas the two \( pK \)'s of the free enzyme, \( K_1 \) and \( K_2 \), are apparent in the \( V/K_m \) data. \( K_2 \) and \( K'_2 \) are the two \( pK \)'s in the \( K_m \) data.

The 2-hydroxy group of the substrate 2-hydroxy-5-nitrophenyl sulphate ionizes with a \( pK \) of 6.4 (Nicholls & Roy, 1971). However, this is unlikely to represent an experimentally determined \( pK \), since experiments conducted with 4-nitrophenyl sulphate as substrate showed molecular \( pK \)'s similar to those observed with 2-hydroxy-5-nitrophenyl sulphate.

The decrease in the \( K_1 \) for sulphate with decreasing pH suggests that the bisulphate anion HSO\(_4^-\) is the true inhibitory species. This interpretation is supported by the independence of the \( K_1 \) for phosphate over the pH range 4.5–5.9, where it exists predominantly as the monoanion H\(_2\)PO\(_4^-\). As H\(_2\)PO\(_4^-\) binding is independent of any residue on the enzyme ionizing over the pH range 4.5–5.9 and assuming that HSO\(_4^-\) and H\(_2\)PO\(_4^-\) bind at the same site (both are competitive inhibitors), then the decrease in \( K_1 \) observed with sulphate over this pH range must be due to protonation of SO\(_4^{2-}\). The \( K_1 \) for HSO\(_4^-\) (calculated for the acetate buffer series) is 0.39 \( \mu \)M for both arylsulphatases A and B. This very tight binding of HSO\(_4^-\) indicates that this species is the true product which normally loses a proton to form SO\(_4^{2-}\) only after diffusing from the enzyme surface.

It is known that (i) hydrolysis of 2-hydroxy-5-nitrophenyl sulphate and 4-nitrophenyl sulphate by arylsulphatase A involves O–S bond fission and (ii) there is no evidence for ‘sulphate’ acceptors, other than water (Spencer, 1958). The latter observation is consistent with a crypto-sequential mechanism where the enzyme, sulphate ester and water form a ternary complex. A consideration of these factors allows us to suggest Scheme 2 as a general model for the mechanism of hydrolysis catalysed by arylsulphatases A and B. Initial binding of the monoanion substrate involves a protonated group (\( pK 5.7–5.8 \)) and a base with a \( pK \) of 4.4–4.5. In the enzyme–substrate complex the \( pK \) of the base is unaltered (\( pK_1 \approx pK'_1 \)), whereas the \( pK \) of the acid group is perturbed significantly by substrate binding (\( pK'_2 > pK_2 \)). The reaction is shown (Scheme 2) as a
concerted mechanism, resulting in the production of the alcohol and bisulphate anion. In the case of arylsulphatase A we suggest that the enzyme-substrate complex occasionally breaks down, not in a concerted fashion, but by an initial protonation of the bridge oxygen and subsequent breakdown of the cation to form sulphur trioxide and the alcohol product. The sulphur trioxide then reacts with a nucleophilic residue to form an inactive species as described previously (O’Fagain et al., 1982). The model we are proposing (i) suggests reasonable roles for the two ionizing groups identified in the present study, (ii) is consistent with (a) the known O–S bond fission, (b) the competitive kinetics exhibited by HSO₄⁻, (c) the apparently crypto-sequential mechanism and (d) the substrate-mediated inactivation of sulphatase A which is thought to involve enzyme-bound sulphur trioxide occasionally sulphating a nucleophilic residue on the protein rather than water (Waheed & Van Etten, 1979; O’Fagain et al., 1982).

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References

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