Aluminofluorides and beryllfluorides as inhibitors of sulphatases

Analogues of hydrogen sulphate?

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The inhibition by fluoride of sulphatase A from ox liver and of the sulphatases of *Helix pomatia* and *Aspergillus oryzae* is decreased by EDTA and increased by Al^{3+} or Be^{2+} , implicating aluminofluorides and beryllfluorides in the reaction. The inhibition, which is reversible, takes several minutes to develop fully and, at least for the sulphatase of *H. pomatia*, is of a non-linear mixed competitive–non-competitive type. It is suggested that the aluminofluorides and beryllfluorides are acting as analogues of HSO_4^- . If so, then this behaviour must be considered, as well as their role as analogues of phosphate, in interpreting the effects of these compounds in intact cells.

INTRODUCTION

It has long been known that fluoride inhibits the glucosulphatase of the marine mollusc *Charonia lampas* (Soda & Egami, 1934) and the arylsulphatase of *Aspergillus oryzae* (Tanaka, 1938), and it now seems that sulphatases are generally subject to such an inhibition. It has been found, with one exception (Dodgson *et al.*, 1955), wherever it has been sought. For example, fluoride inhibits several different types of arylsulphatase (Roy, 1953, 1956b; Dodgson, 1959; Dodgson & Powell, 1959; Webb & Morrow, 1959; Jarrige, 1963; Fowler & Rammler, 1964; Bleszyński & Leznicki, 1967), steroid sulphatases (Roy, 1956a; Jarrige, 1963), cholinesterase (Scott & Spencer, 1968) and heparin sulphamidase (Friedman & Arsenis, 1974). The mechanism of this inhibition is unknown. Roy (1953) suggested that it might reflect a requirement of sulphatase A for Mg^{2+} , by analogy with the well-known effect of fluoride on enolase (Warburg & Christian, 1942), but there was no direct evidence for this hypothesis at that time, and none has since been forthcoming. No metal ion has yet been implicated in sulphatase activity.

Following the observation (Sternweiss & Gilman, 1982) that the activation of adenylate cyclase by fluoride required the presence of Al^{3+} or Be^{2+} , there has been considerable interest in the role of aluminofluorides and beryllfluorides as analogues of phosphate, particularly with G-proteins (Gilman, 1987; Chabre, 1990). These fluoride complexes are also involved in the inhibition of glucose-6-phosphatase (Lange *et al.*, 1986) and of some ATPases (Missiaen *et al.*, 1988) by NaF. As phosphate ions are inhibitors of most, if not all, sulphatases, it seemed that the formation of such aluminofluorides and beryllfluorides might well explain the inhibition of sulphatases by fluoride, and the present paper provides evidence to support this hypothesis.

EXPERIMENTAL

Reagents

The NaF was of AnalaR standard, obtained from BDH Chemicals Pty. Ltd. (Port Fairy, Victoria, Australia), and the required solutions were made up daily to minimize extraction of Al^{3+} from the glass. Solutions of AlCl_3 and BeCl_2 were prepared by dissolving weighed amounts of metallic aluminium or BeCO_3

in 0.1 M-HCl to give 25 mM solutions. These were diluted as required in the reaction mixtures, immediately before use, to give concentrations no greater than $25 \mu\text{M-Al}^{3+}$ or Be^{2+} .

Sulphatases

Sulphatase A and the arylsulphatase of the snail *Helix pomatia* were prepared as previously described (Nichol & Roy, 1964; Roy & Williams, 1989). The specific activities of these preparations were 205 and $30 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ respectively.

The arylsulphatase of *A. oryzae* (takasulphatase) was obtained from Takadiastase (Serva, Heidelberg, Germany). A 10% (w/v) solution of the powder in 0.1 M-sodium acetate, adjusted to pH 6.2 with acetic acid, was run through a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) in the same buffer to remove contaminants of low molecular mass. The active fractions were combined and used without further treatment: the specific activity was about $1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

Determination of arylsulphatase activity

The activity of sulphatase A was measured at pH 5.6 in a pH-stat with 3 mM-2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) at 37 °C in 0.1 M-KCl: the details and the computation of v_0 with the associated parameter k^* , the apparent rate constant for the substrate-induced modification of this enzyme, have been described previously (Roy, 1978).

The arylsulphatase of *H. pomatia* was assayed continuously by monitoring the increase in A_{400} accompanying the liberation of 4-nitrophenol from 20 mM-4-nitrophenyl sulphate in 0.1 M-Tris/HCl, pH 7.4, at 37 °C (Roy & Williams, 1989). For the uninhibited reaction v_0 was measured, but when fluoride was present the rate was measured between 2 and 3 min after the start of the reaction: the justification of the latter procedure is given below.

Takasulphatase activity was measured as described by Robinson *et al.* (1952), with 15 mM-4-nitrophenyl sulphate in 0.5 M-sodium acetate/acetic acid buffer, pH 6.2. After incubation for 1 h at 37 °C, the reaction was stopped by addition of 1 ml of 1 M-NaOH and the liberated 4-nitrophenol was determined spectrophotometrically.

In experiments involving fluoride, with or without Al^{3+} or Be^{2+} , the modifier was incubated with the buffered substrate during temperature equilibration (5 min in the pH-stat and with

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takasulphatase, 20 min in the spectrophotometric assay). The reaction was then started by adding the enzyme. Any alteration to this procedure is detailed below.

RESULTS

The initial observations are given in Table 1. They confirm the classical observations that the arylsulphatase activity of sulphatase A, of the sulphatase of *H. pomatia* and of takasulphatase are inhibited by fluoride. They also show that this inhibition is decreased by EDTA and increased by Al^{3+} or Be^{2+} . These results are consistent with the hypothesis that the inhibition

Table 1. Effect of EDTA and Al^{3+} or Be^{2+} on the inhibition of sulphatases by NaF

The enzyme activities were determined as described in the text and are referred to that of the control reaction as 1.00. With sulphatase A, v_0 was determined; with the sulphatase of *H. pomatia* the inhibited rate was measured between 2 and 3 min after the start of the reaction; with takasulphatase the rate was measured over 1 h.

Modifier(s)	Rate		
	Sulphatase A	<i>H. pomatia</i>	Taka-sulphatase
1 mM-NaF	0.66	0.85	0.96
10 mM-NaF	0.14	0.66	0.88
10 mM-EDTA	0.96	0.85	0.97
+ 1 mM-NaF	0.82	0.91	0.94
+ 10 mM-NaF	0.36	0.88	0.89
25 μM - Al^{3+}	0.92	1.00	0.98
+ 1 mM-NaF	0.04	0.15	0.64
+ 10 mM-NaF	0.00	0.05	0.83
25 μM - Be^{2+}	1.00	1.00	0.99
+ 1 mM-NaF	0.02	0.68	0.56
+ 10 mM-NaF	0.00	0.38	0.42
25 μM - Al^{3+} + 10 mM-EDTA	0.90	0.87	0.96
+ 1 mM-NaF	0.63	0.88	0.92
+ 10 mM-NaF	0.23	0.92	0.83

Fig. 1. Progress curves for the hydrolysis by the sulphatase of *H. pomatia* of 20 mM-4-nitrophenyl sulphate in 0.1 M-Tris/HCl, pH 7.4

■, □, Control reaction. ▲, △, Reaction mixture containing 12.5 μM - Al^{3+} and 0.75 mM-NaF; reaction started by adding enzyme. ●, ○, Reaction mixture containing 12.5 μM - Al^{3+} and 0.75 mM-NaF; reaction started by adding substrate. The points shown by the white symbols were fitted by linear regression to give the rates of hydrolysis in the straight-line portions of the graphs.

Fig. 2. Decrease with time of the rate of hydrolysis of 20 mM-4-nitrophenyl sulphate in 0.1 M-Tris/HCl, pH 7.4, containing 12.5 μM - Al^{3+} and 0.75 mM-NaF, by the sulphatase of *H. pomatia*

The points show the rates measured as described in the text. Those from 15 to 115 s were used, together with the apparent equilibrium rate (dashed line), to calculate the kinetic parameters (Table 2) for the formation of the inhibited enzyme, by eqns. (1) and (2) in the text. The continuous line is drawn with the parameters so obtained. The inset shows the plot of eqn. (1).

is caused by fluoride complexes of the latter ions. The values in Table 1 are means of duplicates, which agreed to within $\pm 5\%$ except where the inhibition was almost complete, for a single set of measurements: a repeat set gave values similarly agreeing with those in Table 1.

Comparison of progress curves for the hydrolysis of nitro-catechol sulphate by sulphatase A, in the presence and absence of 1 mM-NaF, showed that the inhibition developed over several minutes. For the uninhibited reaction, k^* was 0.0045 s^{-1} , in agreement with previous determinations (Roy, 1978), while for the inhibited reaction it was 0.0069 s^{-1} . The difference between these two values must be a measure of the apparent rate constant for the development of the inhibition. No further studies were made with sulphatase A because of the difficulties caused by its complex kinetics (Roy, 1953, 1978), and the arylsulphatase of *H. pomatia* was used for the more detailed investigations reported below.

Arylsulphatase of *H. pomatia*

Progress curves. Progress curves for the hydrolysis of 4-nitrophenyl sulphate in 12.5 μM - Al^{3+} , with and without 0.75 mM-NaF, are shown in Fig. 1. As previously reported (Roy & Williams, 1989), the control reaction was of zero order for about 5 min, after which its velocity very slowly decreased. The progress curve in the presence of fluoride was different: the initial velocity decreased quite rapidly to an almost constant value achieved between 2 and 3 min after zero time. Also shown in Fig. 1 is the progress curve for a reaction started by the addition not of enzyme but of the required amount of solid 4-nitrophenyl sulphate to the buffered enzyme solution, containing 12.5 μM - Al^{3+} and 0.75 mM-NaF, which had been equilibrated for 20 min at 37 °C. In this case, the initial velocity was low, but rose within 3 min to one identical with that of the reaction started by adding enzyme. In the experiments shown in Fig. 1, these two rates, measured between 2 and 3 min after the start of the reaction, were 0.175 and 0.171 A_{400} unit/min respectively. In the latter experiments, the substrate was added as a solid rather than in solution, because the limited solubility of potassium 4-nitrophenyl sulphate would have required the addition of 0.4 ml of its

Fig. 3. Effect of different concentrations of NaF on the hydrolysis by the sulphatase of *H. pomatia* of 4-nitrophenyl sulphate (general conditions as in Fig. 1) in the presence of 12.5 μM - Al^{3+} (a) or 25 μM - Be^{2+} (b)

The rates were measured at zero time ($\square$) or between 2 and 3 min after the start of the reaction ($\circ$). The degree of inhibition is defined as $(v_0 - v)/v_0$, where v_0 was the rate measured in the absence of NaF.

Table 2. Kinetic parameters for inhibition of the arylsulphatase of *H. pomatia* by complex fluorides

The parameters were obtained as described in the text by using aluminofluoride(s) and berylliofluoride(s) as inhibitors at pH 7.4: k_1 and k_2 are apparent first-order rate constants for the development of the inhibition and its reversal respectively, and K is the apparent equilibrium constant k_1/k_2 . The values are means $\pm$ S.D. for six determinations. The concentrations of Al^{3+} and Be^{2+} were 12.5 μM and 25 μM respectively, in 0.75 mM-NaF.

Parameter	Inhibitor	
	Aluminofluoride	Berylliofluoride
K	0.94 ± 0.08	1.07 ± 0.04
$k_1 + k_2$ (s^{-1})	0.018 ± 0.001	0.011 ± 0.001
k_1 (s^{-1})	0.0087 ± 0.0009	0.0057 ± 0.0004
k_2 (s^{-1})	0.0093 ± 0.0003	0.0054 ± 0.0003

solution to the remainder of the reaction mixture (1.6 ml). This considerable change in volume could have displaced the presumed equilibria between the various aluminium complexes and itself have altered the shape of the progress curves. In practice, experiments in which the substrate was added in solution gave results indistinguishable from those in Fig. 1.

Rate of development of the inhibition. Apparent velocity constants for the attainment of the equilibrium between the inhibited and uninhibited forms of the enzyme were obtained from rate measurements of the enzyme reaction during its first 2 min. The inhibited enzyme was assumed to be devoid of activity, an assumption justified by the powerful inhibition at high fluoride concentrations (see Fig. 3 below), and the inhibitor concentration was considered constant (Al^{3+} , 12.5 μM ; enzyme concentration, approx. 10 nM). Measurements of A_{400} of the reaction mixture were made at 10 s intervals from zero time, and the differences between consecutive pairs of readings gave the velocities, and therefore the amount of uninhibited enzyme present, at the midpoints between these two times. Fig. 2 shows the change in these rates with time.

The data were assumed to apply to opposing first-order, or pseudo-first-order, reactions and treated by eqns. (1) and (2), in which a_0 is the initial enzyme concentration and x_e and x are the concentrations of inhibited enzyme at equilibrium and time t respectively (Moore & Pearson, 1981):

$$t = \frac{1}{k_1 + k_2} \ln \frac{x_e}{x_e - x} = \frac{1}{k_1 + k_2} \ln \frac{v_0 - v_e}{v - v_e} \quad (1)$$

$$K = k_1/k_2 = \frac{x_e}{a_0 - x_e} = \frac{v_0 - v_e}{v_e} \quad (2)$$

The second form of each equation expresses these concentrations in terms of reaction velocities and was used in the present situation: the subscripts have the same meaning as before. The values of k_1 and k_2 , the apparent first-order rate constants for the development of the inhibition and its reversal, respectively, and of K are given in Table 2. The possible significance of these parameters is considered below.

Fluoride concentration. The effects of various concentrations of NaF at fixed concentrations of Al^{3+} (12.5 μM) or Be^{2+} (25 μM) on both the initial velocity and that measured between 2 and 3 min after the start of the reaction are shown in Fig. 3. The much lesser effect on the former is obvious. With Al^{3+} , the maximum effect on the latter velocity occurred at a concentration of about 5 mM-NaF [pF 2.3, as defined by Martin (1988)]. With Be^{2+} , the shape of the curve was different and definitely skewed: the maximum inhibition occurred at approx. 1.8 mM-NaF (pF 2.7).

Kinetics of the inhibition. Measurements of the equilibrium rate at different substrate concentrations and fixed concentrations (0–1 mM) of NaF at a constant concentration (12.5 μM) of Al^{3+} showed the inhibition by fluoride to be of the mixed competitive–non-competitive type, because both the apparent K_m and V , determined by the method of Wilkinson (1962), varied with fluoride concentration (Table 3). The competitive component was clearly dominant and, further, was kinetically complex, because K_m varied non-linearly with the concentration of fluoride. The much lesser effect of fluoride concentration on V could have been linear.

DISCUSSION

The results in Table 1 support the hypothesis that the inhibition of sulphatases by fluoride is due to the formation of aluminofluorides or berylliofluorides. In the absence of added Al^{3+} or Be^{2+} , the inhibition must be due to traces of the former coming from the glassware or reagents. The only result in Table 1 which requires comment is that showing an increased concentration of NaF, in the presence of 25 μM - Al^{3+} , giving a decreased inhibition of takasulphatase. This result is consistent with Fig. 3, which shows that over the appropriate range of fluoride concentration an increase in the latter can indeed give a decreased inhibition. The data in Fig. 3 refer to the sulphatase of *H. pomatia*, at pH 7.4, and the position of the corresponding curves for takasulphatase, at pH 6.2, would be expected to be different.

Both Al^{3+} and Be^{2+} form not only binary complexes with F^- but also ternary complexes containing both F^- and OH^- . The proportions of the different species vary greatly with the concentration of F^- in the system (Martin, 1988). Comparison of the curves in the latter reference with those in Fig. 3(a) suggests that the inhibition of the arylsulphatase of *H. pomatia* by aluminofluorides is caused either by $(\text{HO})\text{AlF}_3^-$ or by AlF_3 , or indeed by both. Of these two complexes, the former seems the more likely to be that involved, because arylsulphatases are inhibited by singly charged anions such as HSO_4^- , HSO_3^- and H_2PO_4^- (O'Fagain *et al.*, 1983; Stankiewicz & Gresser, 1988; Roy & Williams, 1989), but a role for AlF_3 cannot be excluded. With

Table 3. Kinetic constants for the inhibition of the arylsulphatase of *H. pomatia* by aluminofluoride(s) at different concentrations of NaF

The enzyme activity at pH 7.4 was determined as described in the text. The concentration of Al^{3+} was held constant at $12.5 \mu\text{M}$. The values of K_m and V were computed by the method of Wilkinson (1962).

[NaF] (mM)	K_m (mM)	V ($\Delta A_{400}/\text{min}$)
0	7.9 ± 0.4	0.548 ± 0.010
0.252	10.7 ± 0.6	0.534 ± 0.013
0.504	17.5 ± 0.7	0.480 ± 0.009
0.755	22.4 ± 1.4	0.475 ± 0.013
1.02	55.3 ± 7.7	0.427 ± 0.038

beryllofluorides the situation is less clear, and the skew curve in Fig. 3(b) suggests that more than one fluoride complex may be inhibitory in this system.

The inhibition by NaF and Al^{3+} or Be^{2+} was rather slow to develop, with both sulphatase A and the sulphatase of *H. pomatia*, and therefore differs from that by sulphate or phosphate which are both classical inhibitors. Only the sulphatase of *H. pomatia* was studied in detail, and the kinetic parameters in Table 2 justify, at least in the case of the aluminofluorides, the use of the rate between 2 and 3 min after the start of the reaction as the equilibrium rate. The values of the velocity constants show that the rate at 150 s would be about 6% greater than the equilibrium value. With beryllofluoride this procedure is less justifiable, the rate at 150 s being about 17% above that at equilibrium, but this does not invalidate the conclusions drawn.

Similarly low rates of development of the inhibition caused by aluminofluorides have been found with several P-type cation-activated ATPases (Missiaen *et al.*, 1988), whereas the reaction of beryllofluorides with F-actin is even slower, perhaps reflecting the rate of a conformational change in the protein (Combeau & Carlier, 1988). The simplest mechanism to explain the present data is that in eqn. (3), in which the rate-limiting reaction is the conversion of EI, a classical enzyme-inhibitor complex, into E^* , an inactive form of the enzyme, which may, but need not necessarily, contain the inhibitor:

A simpler case in which the binding of the inhibitor is itself slow can be excluded, because in such a system the inhibitor would have no effect on the initial velocity: this is contrary to the experimental results (Fig. 3). If the mechanism in eqn. (3) is correct, then the kinetic parameters in Table 2 refer to the reaction $\text{E} \rightleftharpoons \text{E}^*$, but until more information is available about this it will not be possible to define precisely the rate constants.

An assumption implicit in this work, and apparently in all similar studies, is that the rates of equilibration between the Be^{2+} and Al^{3+} complexes is rapid. Information on these rates is scanty, but some data are available for the exchange between $\text{Al}^{3+}\text{aq.}$ and water or sulphate, where the rate constants are of the order of $0.1\text{--}1 \text{ s}^{-1}$ (Burgess, 1978): the rates with fluoride would be expected to be similar. In the present work the Al- and Be-containing solutions were equilibrated for at least 5 min (20 min the case of the sulphatase from *H. pomatia*) before the addition of the enzyme, so it is improbable that attainment of the equilibria between the complexes could influence the development of the inhibition.

The inhibition of the arylsulphatase of *H. pomatia* by

aluminofluoride(s) was mixed competitive-non-competitive, with the former component clearly being dominant (Table 3). Further interpretation was not possible, nor could a value for K_i be obtained because, first, the inhibition was non-linear and, secondly, the concentration of the inhibitory species, or indeed its nature, was unknown and would not vary linearly with the concentration of fluoride (Martin, 1988). The latter may itself explain the non-linear inhibition, although such behaviour does not seem to have been found with other enzymes, for example with glucose-6-phosphatase (Lange *et al.*, 1986). Although K_i cannot be obtained, its value must be less than $1 \mu\text{M}$, because 90% inhibition is caused (Fig. 3) by NaF in $12.5 \mu\text{M}\text{-Al}^{3+}$, and the latter must be greater, probably considerably greater (Martin, 1988), than the concentration of the inhibitor.

It is generally accepted that aluminofluorides and beryllofluorides are analogues of phosphate, because the length of the Al-F bond [0.15–0.16 nm (1.5–1.6 Å); Chabre, (1990)] is similar to that of the P-O bond [0.154 nm (1.54 Å); Pauling (1960)], and it would be interesting to know whether their inhibition of sulphatases is a further reflection of this property. Phosphate is only a rather weak inhibitor of the sulphatase of *H. pomatia* (Dodgson & Powell, 1959), and is a non-competitive inhibitor, with a K_i of 20 mM at pH 7.5 (A. B. Roy, unpublished work); therefore, if the fluoride complexes are analogues of phosphate, the affinity of the enzyme for the analogue is about four orders of magnitude greater than for phosphate itself. A similar difference has been noted with F-actin (Combeau & Carlier, 1988).

However, the possibility exists that in the present instance the aluminofluoride(s) and beryllofluoride(s) are analogues, not of phosphate (H_2PO_4^-) but of hydrogen sulphate (HSO_4^-). Sulphur and phosphorus atoms are similarly sized, and the bond lengths for P-O and S-O are little different [0.154 nm (1.54 Å) and 0.149 nm (1.49 Å) respectively; Pauling (1960)]. Further, HSO_4^- is, unlike H_2PO_4^- but like aluminofluoride, a competitive inhibitor of the arylsulphatase of *H. pomatia* (Roy & Williams, 1989). At pH 7.5, the apparent K_i is about 1.5 M-sulphate, but, as the inhibitory species is certainly HSO_4^- , then the true K_i is about $4 \mu\text{M}\text{-HSO}_4^-$, of the same order as that for aluminofluoride.

Should aluminofluorides and beryllofluorides indeed be analogues of HSO_4^- as well as of H_2PO_4^- , then it is clear that this must be taken into account when interpreting their effects on intact cells. It would be particularly interesting to know their effect on ATP-sulphurylase, although the requirement of this enzyme for Mg^{2+} could complicate the interpretation of the results.

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