Se-Aryl selenenylthiosulphates and S-aryl sulphenylthiosulphates as thiol-blocking reagents

Nuansri PATARASAKULCHAI and Peter T. SOUTHWELL-KEELY

Department of Organic Chemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033, Australia

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Se-Aryl selenenylthiosulphates and S-aryl sulphenylthiosulphates inhibit papain at pH 5.8 much more rapidly than do the corresponding Se-aryl selenosulphates and S-aryl thiosulphates, and also more rapidly than do Se-alkyl selenosulphates. Se-*p*-Nitrophenyl selenenylthiosulphate and S-*p*-nitrophenyl sulphenylthiosulphate inactivate papain most rapidly, but the inactivation is slowly and spontaneously reversible. Inactivation by Se-*o*-nitrophenyl selenenylthiosulphate and S-*o*-nitrophenyl sulphenylthiosulphate, although less rapid than that by the *para* isomers, is essentially irreversible.

Se-Alkyl selenosulphates have been shown to be very active thiol-blocking reagents, whereas their sulphur isologues are completely inactive at much higher concentrations (Scarf *et al.*, 1982). The selenosulphates show the characteristics of reversible binding followed by irreversible reaction of bound inhibitor with glutathione reductase (EC 1.6.4.2), although gel-permeation chromatography of the inactivated enzyme, or of similarly treated papain (EC 3.4.22.2), restores enzyme activity.

A logical extension of this work was to examine the reactivities of selenenylthiosulphates and to compare them with those of sulphenylthiosulphates. Since the selenenylthiosulphates are known to react with thiols in a similar manner to that of the selenosulphates, e.g.:

(N. Patarasakulchai & P. T. Southwell-Keely, unpublished work) and since thiosulphate

(S₂O₃²⁻) is a weaker S-base (and therefore a better leaving group) than sulphite (Parker & Kharasch, 1959; Parker, 1962), so selenenylthiosulphates should react more rapidly than the selenosulphates with thiol-dependent enzymes.

Experimental

Materials

The following materials were obtained commercially: α -N-benzoyl-L-arginine ethyl ester hydrochloride, dithiothreitol (both from Sigma Chemical Co., St. Louis, MO, U.S.A.), papain (water-soluble; 3.5×10^{-3} Anson units/mg), 5,5'-dithiobis-(2-nitrobenzoic acid) (both from E. Merck, Darmstadt, Germany), N-ethylmaleimide, potassium thiosulphate, *o*-nitroaniline, *p*-nitroaniline, *o*-chloronitrobenzene (all from BDH Chemicals, Melbourne, Vic., Australia), potassium sulphite (Fisher Scientific, Fairlawn, NJ, U.S.A.), *p*-chloronitrobenzene (Tokyo Kasei, Tokyo, Japan), sodium sulphide nonahydrate (Ajax Chemicals, Sydney, N.S.W., Australia), Sephadex G-25, Blue Dextran 2000 (both from Pharmacia, Uppsala, Sweden) and *p*-nitrobenzyl bromide (Eastman Kodak, Rochester, NY, U.S.A.). *p*NO₂PhCH₂-S-SO₃⁻K⁺ and *p*NO₂PhCH₂-Se-SO₃⁻K⁺ were prepared as described previously (Scarf *et al.*, 1977). *o*NO₂Ph-Se-SO₃⁻K⁺, *o*NO₂Ph-S-SO₃⁻K⁺, *o*NO₂Ph-Se-S-SO₃⁻K⁺, *o*NO₂Ph-S-S-SO₃⁻K⁺, *p*NO₂Ph-S-SO₃⁻K⁺ and *p*NO₂Ph-S-S-SO₃⁻K⁺ were prepared to satisfactory purity by the reaction of the selenenyl bromide, R-Se-Br, or the sulphenyl chloride, R-S-Cl, with sulphite or

Abbreviations used: *p*NO₂PhCH₂-S-SO₃⁻K⁺, potassium S-*p*-nitrobenzyl thiosulphate; *p*NO₂PhCH₂-Se-SO₃⁻K⁺, potassium Se-*p*-nitrobenzyl selenosulphate; Ph-Se-S-SO₃⁻K⁺, potassium Se-phenyl selenenylthiosulphate; *o*NO₂Ph-Se-SO₃⁻K⁺, potassium Se-*o*-nitrophenyl selenosulphate; *o*NO₂Ph-S-SO₃⁻K⁺, potassium S-*o*-nitrophenyl thiosulphate; *o*NO₂Ph-Se-S-SO₃⁻K⁺, potassium Se-*o*-nitrophenyl selenenylthiosulphate; *o*NO₂Ph-S-S-SO₃⁻K⁺, potassium S-*o*-nitrophenyl sulphenylthiosulphate; *p*NO₂Ph-S-SO₃⁻K⁺, potassium S-*p*-nitrophenyl thiosulphate; *p*NO₂Ph-S-S-SO₃⁻K⁺, potassium S-*p*-nitrophenyl sulphenylthiosulphate; *p*NO₂Ph-Se-SO₃⁻K⁺, potassium Se-*p*-nitrophenyl selenosulphate; *p*NO₂Ph-Se-S-SO₃⁻K⁺, potassium Se-*p*-nitrophenyl selenenylthiosulphate.

thiosulphate (Foss, 1947). For the preparation of $\text{Ph-Se-S-SO}_3^- \text{K}^+$, $p\text{NO}_2\text{Ph-Se-SO}_3^- \text{K}^+$ and $p\text{NO}_2\text{Ph-Se-S-SO}_3^- \text{K}^+$, a modification of the above method was used in which dry chloroform instead of benzene was employed as a solvent for the selenenyl bromides and the reaction was carried out at 5°C for 30 min instead of room temperature for 2 h.

Methods

Enzyme assays were performed with a Cary 17 spectrophotometer thermostatically controlled with a Haake NK 22 water bath.

Ultrafiltration was performed with a UM 10 membrane in a 50 ml cell (Amicon Corp.) under N_2 at 345 kPa (50 lbf/in²). Gel permeation chromatography was performed at $3-4^\circ\text{C}$ on Sephadex G-15 columns (2 cm $\times$ 30 cm).

Papain was activated and its activity assayed as described previously (Scarf *et al.*, 1982).

Results

Fig. 1 illustrates the rates of inactivation of papain by the above selenosulphates, selenenylthiosulphates, thiosulphates and sulphenylthiosulphates, and also, for comparison, by 5,5'-dithio-bis-(2-nitrobenzoic acid). Papain was rapidly inactivated (more than 90%) within 1 min by $p\text{NO}_2\text{Ph-Se-S-SO}_3^-$ and $p\text{NO}_2\text{Ph-S-S-SO}_3^-$, but the inactivation was slowly and spontaneously reversible and within 15 min activity had returned

to 26% and 18% respectively of the control. Inactivation by $o\text{NO}_2\text{Ph-Se-S-SO}_3^-$ and $o\text{NO}_2\text{Ph-S-S-SO}_3^-$ was less rapid but was more complete than in the case of the *para* isomers and showed no tendency towards spontaneous reversibility. Each of the selenenylthiosulphates and sulphenylthiosulphates reacted much more rapidly with papain than did the corresponding selenosulphates and thiosulphates, and considerably faster than the $p\text{NO}_2\text{PhCH}_2\text{-Se-SO}_3^-$ described previously (Scarf *et al.*, 1982). $p\text{NO}_2\text{PhCH}_2\text{-Se-SO}_3^-$ reacted much more rapidly with papain than did $p\text{NO}_2\text{Ph-Se-SO}_3^-$.

Since $o\text{NO}_2\text{Ph-Se-S-SO}_3^-$ and $o\text{NO}_2\text{Ph-S-S-SO}_3^-$ were the strongest inhibitors of papain, tests were made of the reversibility of their inhibition. Plots of V (velocity at saturating concentration of substrate) versus $[E]_t$ (where $[E]_t$ is the total amount of enzyme present with or without added inhibitor; Segel, 1975) revealed behaviour typical of irreversible inhibitors (Fig. 2).

Reaction of $o\text{NO}_2\text{Ph-Se-S-SO}_3^-$ and $o\text{NO}_2\text{Ph-S-S-SO}_3^-$ with papain for 15 min followed by gel-permeation chromatography to remove excess of inhibitor (Table 1) showed that chromatography had effected a slight re-activation in the case of $o\text{NO}_2\text{Ph-Se-S-SO}_3^-$ but almost no re-activation in the case of the $o\text{NO}_2\text{Ph-S-S-SO}_3^-$ reaction. When these inactivated enzymes were treated with dithiothreitol, full reactivity was restored in each case, demonstrating that inactivation had been due to reaction with the thiol group of the enzyme.

Fig. 1. Time-dependent inhibition of papain by selenosulphates/thiosulphates/selenenylthiosulphates/sulphenylthiosulphates. Activated papain (0.6 unit; specific activity 0.57 unit/mg) was incubated at 30°C with inhibitor (60 nmol) in 0.25 M-sodium acetate buffer, pH 5.8 (containing $13 \mu\text{M}$ -EDTA and adjusted to $I 0.3$ with KCl) (0.2 ml). At the appropriate time the enzyme-catalysed reaction was started by addition of a portion of the reaction mixture (0.1 ml) to α -N-benzoyl-L-arginine ethyl ester hydrochloride ($4.08 \mu\text{mol}$) in 0.25 M-sodium acetate buffer, pH 5.8 (2.9 ml). Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

Fig. 2. Plot of V (velocity at saturating concentration of substrate) versus $[E]_t$ [total enzyme present] for papain in the presence of $o\text{NO}_2\text{Ph-Se-S-SO}_3^- \text{K}^+$ and $o\text{NO}_2\text{Ph-S-S-SO}_3^- \text{K}^+$.

Activated papain (0.6–1.75 units; specific activity 0.57 unit/mg) was incubated at 30°C for 10 min with inhibitor (60 nmol) in 0.25 M-sodium acetate buffer, pH 5.8 (containing 13 μM-EDTA and adjusted to 10.3 with KCl) (0.2 ml). The reaction was started by addition of a portion of the reaction mixture (0.1 ml) to α -N-benzoyl-L-arginine ethyl ester hydrochloride (4.08–12.24 μmol) in 0.25 M-sodium acetate buffer, pH 5.8 (2.9 ml). ●, Control without inhibitor; □, $o\text{NO}_2\text{Ph-Se-S-SO}_3^- \text{K}^+$ (10 μM); ○, $o\text{NO}_2\text{Ph-S-S-SO}_3^- \text{K}^+$ (10 μM).

Table 1. Reversibility of selenenylthiosulphate/sulphenylthiosulphate inhibition of papain by dithiothreitol

Activated papain (6 units; specific activity 0.53 unit/mg) was treated with $o\text{NO}_2\text{Ph-Se-S-SO}_3^- \text{K}^+$ (0.6 μmol), $o\text{NO}_2\text{Ph-S-S-SO}_3^- \text{K}^+$ (0.6 μmol) or *N*-ethylmaleimide (0.6 and 3.3 μmol) at 30°C for 15 min in 0.25 M-sodium acetate buffer, pH 5.8 (containing 13 μM-EDTA and adjusted to 10.3 with KCl) (2 ml), and then excess of inhibitor was removed by gel-permeation chromatography (see the Experimental section) and the activity analysed in the usual manner. A control was performed to account for activity loss due to oxidation during gel-permeation chromatography of papain alone. The inactivated papain was then treated with dithiothreitol (0.8 mM) at 20°C for 10 min and the activity analysed as before. All activities are expressed as percentages of the control. (a) Activity after treatment with inhibitor; (b) activity after treatment with inhibitor and then gel-permeation chromatography; (c) activity after (b) and then treatment with dithiothreitol.

Inhibitor	Papain activity		
	(a)	(b)	(c)
$o\text{NO}_2\text{Ph-Se-S-SO}_3^- \text{K}^+$	13.9	27.6	100
$o\text{NO}_2\text{Ph-S-S-SO}_3^- \text{K}^+$	2.9	6.5	100
<i>N</i> -Ethylmaleimide (0.6 μmol)	83.1	83.5	85.7
<i>N</i> -Ethylmaleimide (3.3 μmol)	17.4	11.9	24.1

Discussion

Scarf *et al.* (1982) showed that the concentrations of *Se*-benzyl selenosulphate ($\text{PhCH}_2\text{-Se-SO}_3^- \text{K}^+$) and $p\text{NO}_2\text{PhCH}_2\text{-Se-SO}_3^- \text{K}^+$ required to inhibit glutathione reductase and papain made them among the most active thiol-blocking inhibitors. It is clear from the present results (Fig. 1) that $o\text{NO}_2\text{Ph-Se-S-SO}_3^-$ and $o\text{NO}_2\text{Ph-S-S-SO}_3^-$ are even more active thiol-blocking reagents.

Although selenosulphates react more rapidly with thiols than do thiosulphates, selenenylthiosulphates and sulphenylthiosulphates react at much the same rate. This may be explained as follows:

In inorganic thiosulphate ($\text{S}_2\text{O}_3^{2-}$) there is a significant amount of double-bond character between the central and peripheral sulphur atoms (Cotton & Wilkinson, 1980). On the basis of their relative atomic sizes it would be expected that π -bonding would be more favourable in the case of thiosulphates than for selenosulphates, and consequently that structure (II) would make a stronger contribution to the thiosulphates than to the selenosulphates. As any tendency towards multiple bonding must make the bond harder to break, this may explain the lesser reactivity of the thiosulphates than selenosulphates towards thiols. Although it is known that 'X' may also conjugate with the aromatic ring 'Ar' (as discussed below), it may be that its ability to conjugate with sulphite is more important.

By contrast, with the selenenylthiosulphates and sulphenylthiosulphates the π -bonding of the additional sulphur atom with terminal sulphite would enhance the leaving potential of the thiosulphate group equally for both compounds:

In addition, the ability of 'X' to conjugate with the nitro aromatic ring would have the effect of weakening the central X-S bond, making both X

reactions were considerably faster for these compounds than for $p\text{NO}_2\text{PhCH}_2\text{-Se-SO}_3^-$.

It is interesting to note that $p\text{NO}_2\text{PhCH}_2\text{-Se-SO}_3^-$ inactivates papain significantly faster than does $p\text{NO}_2\text{Ph-Se-SO}_3^-$.

The contribution of (X) to the structure of (IX) would render a nucleophilic displacement more difficult by tending to make it a two-step reaction (9) rather than a one-step reaction (10):

5,5'-Dithiobis-(2-nitrobenzoic acid) has been employed both to determine and to inhibit thiol groups of enzymes (Ellman, 1959; Brocklehurst, 1979), and it reacts with them to form mixed disulphides similar to that formed by $p\text{NO}_2\text{Ph-S-SO}_3^-$ except that the derivative formed with 5,5'-dithiobis-(2-nitrobenzoic acid) has a carboxylic group in the position *ortho* to the nitro group.

The fact that both the selenenylthiosulphates and the sulphenylthiosulphates react much more rapidly with papain than does 5,5'-dithiobis-(2-nitrobenzoic acid) is probably because $\text{S}_2\text{O}_3^{2-}$ is a weaker S-base and therefore a better leaving group than 4-nitro-5-carboxythiophenol. $\text{S}_2\text{O}_3^{2-}$ is known to be a better leaving group than 4-nitrothiophenol (Parker & Kharasch, 1959) and would be expected to be a better leaving group than 4-nitro-5-carboxythiophenol.

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