Alkyl selenosulphates (seleno Bunte salts)

A new class of thiol-blocking reagents

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Potassium benzyl selenosulphate and potassium *p*-nitrobenzyl selenosulphate were shown to be powerful inhibitors of the thiol-dependent enzymes glutathione reductase and papain, but to have no effect on the serine-dependent proteinase trypsin. By contrast, potassium benzyl thiosulphate and potassium *p*-nitrobenzyl thiosulphate, at much higher concentrations, have virtually no effect on any of the enzymes. The selenosulphates show characteristics of both reversible non-competitive and irreversible inhibition. On the basis of model reactions in which the selenosulphates react instantly with cysteine, it is suggested that they form labile selenosulphide derivatives with the enzymes, but that these derivatives may be broken down either by the normal functioning of the enzyme (in the case of glutathione reductase) or by the approaching substrate (in the case of papain). Continued inhibition of the enzymes requires a stoichiometric excess of inhibitor over enzyme.

The reactivity of the thiol groups of enzymes and other biological materials has led to the use of a range of modifying agents giving covalently bound groups (Brocklehurst, 1979). It has been shown that alkyl selenosulphates (RSeSO_3^-) also have the potential to be good thiol-blocking agents (Scarf *et al.*, 1977, 1979). Although both alkyl thiosulphates (RSSO_3^-) and selenosulphates react with cysteine (CySH) according to eqn. (1) ($\text{X} = \text{S}$ or Se), products appear in different proportions and reaction with selenosulphates is clearly faster (Scarf *et al.*, 1977):

Studies on antimicrobial activity have also shown marked differences in effects where, contrasting with the virtual inactivity of the thiosulphates, the selenosulphates show good potential as antifungal agents (Scarf *et al.*, 1979).

Comparative reactivities have now been examined further with respect to ability to inhibit the thiol-group-dependent enzymes glutathione reductase (EC 1.6.4.2) and papain (EC 3.4.22.2).

Abbreviations used: $\text{BzISeSO}_3^- \text{K}^+$, potassium benzyl selenosulphate; $\text{NO}_2\text{BzISeSO}_3^- \text{K}^+$, potassium *p*-nitrobenzyl selenosulphate; GSSG, oxidized glutathione; GSH, reduced glutathione; BzArgOEt , α -*N*-benzoyl-L-arginine ethyl ester hydrochloride.

Experimental

Materials

The following materials were obtained commercially: glutathione reductase (type III from yeast), GSSG (type III), NADPH (tetrasodium salt), dithiothreitol, bovine serum albumin, BzArgOEt (all Sigma Chemical Co., St. Louis, MO, U.S.A.), papain (water-soluble; 3.5-m-Anson units/mg) (E. Merck, Darmstadt, Germany), trypsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.), *N*-ethylmaleimide (BDH Chemicals, Melbourne, Vic., Australia), potassium sulphite (Fisher Scientific, Fair Lawn, NJ, U.S.A.), benzyl bromide (Fluka A.G., Buchs, Switzerland), *p*-nitrobenzyl bromide (Eastman Kodak, Rochester, NY, U.S.A.), Sephadex G-15 and Blue Dextran 2000 (Pharmacia, Uppsala, Sweden). Preparation and purification of $\text{BzISeSO}_3^- \text{K}^+$, $\text{NO}_2\text{BzISeSO}_3^- \text{K}^+$, potassium benzyl thiosulphate and potassium *p*-nitrobenzyl thiosulphate were as described previously (Scarf *et al.*, 1977; Price & Jones, 1909).

Instrumental methods

Enzyme assays were performed with a Cary 17 spectrophotometer thermostatically controlled with a Haake NK22 water bath. All other u.v.–visible-absorption measurements were made with a Perkin–Elmer 124 double-beam spectrophotometer.

Ultrafiltration was performed with a UM10 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°C on Sephadex G-15 columns (2 cm × 30 cm).

Protein concentrations of the enzymes were determined by absorbance at 280 nm by using for glutathione reductase $A_{1\text{cm}}^{1\%} = 18.6$ (Massey & Williams, 1965), for papain $\epsilon = 5.1 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Glazer & Smith, 1961) and for trypsin $A_{1\text{cm}}^{1\%} = 17.1$ (Kunitz, 1947).

Thiol groups of enzymes were determined by titration with 5,5'-dithiobis-(2-nitrobenzoic acid) (Janatova *et al.*, 1968).

Glutathione reductase activity was measured by oxidation of NADPH, with either GSSG or NADPH being used to initiate the reaction (Mize & Langdon, 1962).

Papain activity was assayed spectrophotometrically by hydrolysis of BzArgOEt (Whitaker & Bender, 1965), after activation of enzyme (40 mg) with dithiothreitol (8 mg) at 30°C in 0.4 ml of 0.1 M-Tris/HCl buffer, pH 7.5, containing 10 mM-EDTA for 1 h, removal of dithiothreitol by gel-permeation chromatography and concentration of the enzyme solution by ultrafiltration.

Trypsin activity was also assayed by hydrolysis of BzArgOEt (Schwert & Takenaka, 1955). The unit of enzyme activity is defined as the amount of enzyme that transforms 1 μmol of substrate (GSSG or BzArgOEt)/ml per min.

Results

Experiments with glutathione reductase

Inhibition by the selenosulphates was dependent on the order of addition of NADPH, inhibitor and GSSG, and was only apparent when the enzyme was preincubated with the selenosulphates in the presence of NADPH, and the reaction started by addition of GSSG. Inhibition was not evident when the enzyme was preincubated with the selenosulphates in the presence of GSSG and the reaction started by addition of NADPH.

Since the thiosulphates were very weak inhibitors even at much higher concentrations, analysis of their reactions with glutathione reductase was not carried further.

Kinetic analysis of inhibition reaction. Although Lineweaver–Burk plots and plots of V_{max} versus E_{total} (where E_{total} is the total amount of enzyme present with or without added inhibitor; Segel, 1975) revealed behaviour typical of reversible non-competitive inhibition for the selenosulphates, a test of irreversible inhibition was made.

In the following scheme for the reaction of an enzyme with an irreversible inhibitor (Main, 1973)

E:I represents a reversible complex between enzyme (E) and inhibitor (I), with a dissociation constant $K_a = k_{-1}/k_{+1}$, and E–I is the irreversible complex, formed with first-order rate constant k_{+2} . k_i is the rate constant for the simple bimolecular reaction between E and I in which E:I is not formed, and is only applicable where $[\text{I}] \ll K_a$. It has been demonstrated that, on incubation with a large excess of an irreversible inhibitor, enzymes show a first-order loss of activity with time and that a plot of $[\text{i}]/\rho$ versus $[\text{i}]$ (eqn. 2)

$$\frac{[\text{i}]}{\rho} = \frac{[\text{i}]}{k_{+2}} + \frac{K_a}{k_{+2}} \quad (2)$$

(where $[\text{i}]$ is inhibitor concentration and ρ is the first-order rate constant of inactivation) gives a straight line. In the case of this plot, a significant slope indicates that formation of the irreversible complex between enzyme and inhibitor is preceded by formation of a reversible complex (Aldridge, 1950; Kitz & Wilson, 1962; Main, 1973).

By this criterion both selenosulphates are irreversible inhibitors (Figs. 1 and 2), with formation of the

Fig. 1. Time-dependent inhibition of glutathione reductase by alkyl selenosulphates

Glutathione reductase (0.01 unit) was incubated at 37°C with NADPH (0.45 μmol), selenosulphate (0.6 nmol), bovine serum albumin (0.1 mg) and Tris/HCl buffer, pH 7.5 (0.125 mmol), in a volume of 2.6 ml. At the appropriate time the reaction was started by addition of GSSG (1.5 μmol in 0.4 ml). O, BzlSeSO₃⁻K⁺ (0.2 μM); ■, NO₂BzlSeSO₃⁻K⁺ (0.2 μM).

irreversible enzyme-inhibitor complex preceded in each case by formation of a reversible complex.

The calculated rate constants (Table 1) show that the affinity (K_a) of $\text{NO}_2\text{BzlSeSO}_3^-$ for glutathione reductase is 8-fold smaller than that of BzlSeSO_3^- . By contrast, k_{+2} values for both selenosulphates are very similar.

Gel-permeation chromatography of the products of the inhibition reaction. The products of a 15 min reaction between glutathione reductase and the selenosulphates (with or without NADPH) were submitted to gel-permeation chromatography. Table 2 shows that, in the absence of NADPH, both

$\text{NO}_2\text{BzlSeSO}_3^-$ and *N*-ethylmaleimide were moderate inhibitors of comparable activity, whereas BzlSeSO_3^- had only slight activity. However, in the presence of NADPH, *N*-ethylmaleimide was almost totally inhibitory, but the activities of both selenosulphates were little different from the control. The fact that inhibition by the selenosulphates can be removed by gel-permeation chromatography is further support for the theory of reversible inhibition by these compounds, whereas *N*-ethylmaleimide shows true irreversible inhibition.

Inhibition-re-activation studies. Glutathione reductase was allowed to react with BzlSeSO_3^- (20 μM) and $\text{NO}_2\text{BzlSeSO}_3^-$ (20 μM) at 25°C until inhibition had reached 85% and 75% respectively (50 min), and dithiothreitol (0.4 mM) was then added. Activity was rapidly regained, and after a further 60 min was 84% and 78% respectively of the initial activity. Evidence is thus provided for the reaction by the selenosulphates with enzyme thiol groups.

Experiments with papain

Further support for the thiol-blocking role of selenosulphates was sought in their reaction with papain, a proteolytic enzyme containing only one thiol group per molecule and that at its active site (Lowe, 1976).

Kinetic analysis of the inhibition reaction. The Lineweaver-Burk plot for $\text{NO}_2\text{BzlSeSO}_3^-$ against papain again showed the characteristics of reversible non-competitive inhibition, as did the plot of V_{max} versus E_{total} .

Gel-permeation chromatography of the products of the inhibition reaction. Gel-permeation chromatography of the products of the reaction between papain and $\text{NO}_2\text{BzlSeSO}_3^-$ gave results analogous to those for the glutathione reductase reaction (Table 3). Thus, after gel-permeation chromatography, the *N*-ethylmaleimide-inhibited enzyme exhibited only 26% of its original activity, whereas the $\text{NO}_2\text{BzlSeSO}_3^-$ -inhibited enzyme contained 88% of its original activity. These results also support the theory of reversible inhibition by the selenosulphates.

Fig. 2. Plot of ρ values (rate constants for inactivation) of the selenosulphates at 25°C according to eqn. (2). Curves for time-dependent inhibition of glutathione reductase with increasing concentration of selenosulphates were obtained as in Fig. 1 and the results are plotted according to eqn. (2). $\circ$, $\text{BzlSeSO}_3^- \text{K}^+$; $\blacksquare$, $\text{NO}_2\text{BzlSeSO}_3^- \text{K}^+$.

Table 1. Inhibitor constants at 25°C for the reaction of selenosulphates with glutathione reductase derived from Fig. 2

Constant	$\text{NO}_2\text{BzlSeSO}_3^- \text{K}^+$	$\text{BzlSeSO}_3^- \text{K}^+$
K_a	$4.05 \times 10^{-5} \text{ M}$	$4.86 \times 10^{-6} \text{ M}$
k_1	$3.45 \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$	$3.33 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$
k_{+2}	0.14 min^{-1}	0.16 min^{-1}

Table 2. Reversibility of selenosulphate inhibition of glutathione reductase by gel-permeation chromatography. Glutathione reductase (50 μg) was treated at 37°C with inhibitor (0.04 μmol) in the presence or in the absence of NADPH (66 μmol) in 0.1 M-Tris/HCl buffer, pH 7.5 (0.15 ml), for 15 min, then excess of inhibitor and NADPH were removed by gel-permeation chromatography (see the Experimental section) and the activity was analysed in the usual manner. Controls without inhibitor, with and without NADPH, were treated in the same way. The activity of the control preincubated without NADPH was taken as 100%.

	Glutathione reductase activity (%)			
	Control	$\text{BzlSeSO}_3^- \text{K}^+$	$\text{NO}_2\text{BzlSeSO}_3^- \text{K}^+$	<i>N</i> -Ethylmaleimide
Enzyme	100	86	64	65
Enzyme + NADPH	65	80	50	4

Experiments with trypsin

The ability of the selenosulphates to inhibit trypsin was studied because trypsin is a proteolytic enzyme with a similar mechanism of action to papain (Huber & Bode, 1978) but contains no free thiol groups (Light & Sinha, 1967).

Neither the selenosulphates nor *N*-ethylmaleimide at a concentration of 250 μ M inhibited trypsin activity.

Discussion

The ability of the selenosulphates to react rapidly with cysteine (Scarf *et al.*, 1977) and to inhibit the thiol-dependent enzymes glutathione reductase and papain, and the removal of such inhibition by thiols, together with their inability to inhibit the thiol-

deficient enzyme trypsin, is strong evidence for their mode of action being that of thiol inhibition. In addition, the selenosulphates have shown no tendency to react with histidine, tryptophan or tyrosine. The possibility of a non-specific, non-covalent, hydrophobic interaction between the enzymes and the selenosulphates is ruled out by the fact that the isologous thiosulphates have no inhibitory activity.

The greater reactivity of the selenosulphates than thiosulphates correlates directly with their respective reactivities against cysteine (Scarf *et al.*, 1977), and as antimicrobial agents (Scarf *et al.*, 1979).

Time-dependent loss of activity of glutathione reductase on incubation with the selenosulphates shows the characteristics of active-site-directed irreversible inhibition. However, when glutathione reductase and papain were treated with selenosulphates and the excess of inhibitor was removed by gel-permeation chromatography, both enzymes regained their activity. This behaviour is typical of reversible inhibitors.

In the case of papain the explanation of this inhibitory activity that fits the data best is that the selenosulphates react rapidly to form a selenophide derivative with the enzyme:

This derivative may be sufficiently labile to hydrolysis to regenerate the enzyme at a reasonable rate:

Table 3. Reversibility of selenosulphate inhibition of papain by gel-permeation chromatography

Activated papain (0.07 unit) was allowed to react with inhibitor (0.12 μ mol) at pH 5.8 for 30 min at 27°C, then excess of inhibitor was removed by gel-permeation chromatography (see the Experimental section) and the activity was analysed in the usual manner.

Papain activity (%)		
Control	<i>N</i> -Ethylmaleimide	$\text{NO}_2\text{BzlSeSO}_3^-\text{K}^+$
100	26	88

Scheme 1

[Hydrolysis of selenosulphides is known to yield the thiol and selenenic acid (Rheinboldt & Giesbrecht, 1950).] As long as an excess of selenosulphate is present, reaction (3) could re-occur, maintaining the thiol bound as Enz-S–SeR by mass-action effect. However, on removal of excess of selenosulphate, as by gel-permeation chromatography, reaction (4) would then proceed, leading to regeneration of activity. A similar explanation may be given for the more complicated case of glutathione reductase.

The mechanism of the normally functioning enzyme is believed to follow the sequence (I)–(IV) (Scheme 1). NADPH first reduces the oxidized enzyme (I) to (II), containing the flavin thiolate charge-transfer complex and a free thiol (Williams, 1976; Schulz *et al.*, 1978), and GSSG reacts with (II) to form (III), (IV) and subsequently (I), liberating 2 mol of GSH/mol. However, it is possible that (II) may react with the selenosulphate to form Enz-S–SeR (V), which may in turn be attacked by the charge-transfer thiolate to regenerate active enzyme and eject RSeH.

Again, an equilibrium may be envisaged in which glutathione reductase is maintained in the form Enz-S–SeR (V) while excess of selenosulphate is present.

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