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CHEMICAL MODIFICATION OF RHODANESE WITH SULPHITE

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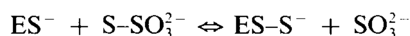
The essential sulphydryl group of bovine liver rhodanese (thiosulphate: cyanide sulphurtrasferase, E.C. 2.8.1.1.) is modified by sulphite produced during the enzymatic reaction or added to the fully active enzyme. The enzyme treated with labelled reagent incorporates 1 equivalent of SO_3^{2-} and loses one -SH group with the formation of a S-sulphonate group at the active site. Mercaptoethanol is effective in both restoring enzyme activity and removing bound sulphite from protein. The inactivation process is dependent on the presence of oxygen and is antagonized by chelation of metal ions, that catalyze sulphite autoxidation, or by scavenging free radicals with mannitol or benzoate. Since the presence of superoxide dismutase and/or catalase protects the enzyme only to a small extent, the inactivation process should be attributed to sulphite radicals rather than intermediates of oxygen reduction.

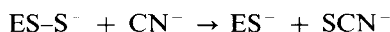
KEY WORDS: Rhodanese, sulphite, free radical, S-sulfonate.

INTRODUCTION

Rhoadanese (thiosulphate: cyanide sulphurtransferase, EC 2.8.1.1.) catalyzes sulphur transfer between thiosulphate and cyanide with release of sulphite and thiocyanate. The reaction occurs by a double displacement mechanism with the formation of an intermediate enzyme-sulphur complex:[1,2]



$$ES^- + S\text{-}SO_3^{2-} \Leftrightarrow ES\text{-}S^- + SO_3^{2-}$$



$$ES\text{-}S^- + CN^- \rightarrow ES^- + SCN^-$$

In the first half reaction the so-called sulphur-free enzyme (ES^-) binds reversibly the outer sulphur of thiosulphate on the cys-247 as a persulphide group ($ES\text{-}S^-$) releasing sulphite. Sorbo found that an excess of sulphite rapidly inactivates the enzyme and interpreted this process as being due to a reaction of sulphite with a disulphide bond in the enzyme.[3] The lack of disulphide in the native enzyme and the dependence of the inactivation on the presence of oxygen induced later the same author to invoke autoxidation of the essential sulphydryl group of the enzyme for explaining the binding of sulphite as thiosulphate ester ($ES\text{-}SO_3^-$) to the inactive enzyme.[4] More recently Volini reports that the reaction of sulphur-free enzyme with sulphite give rise

This paper is dedicated to Professor Doriano Cavallini on the occasion of his 75th birthday.

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to a catalytically inert conformer in which the conformational change accompanies the formation of the E–SO_3^{2-} ion pair.[5] It is also known that sulphite solutions autoxidizes readily to sulphate in air at physiological pH values and temperature.[6] This autoxidation is a free radical chain reaction which may be initiated by any process that affects either the univalent oxidation of sulphite or univalent reduction of oxygen.[7] We recently demonstrated that the essential sulphydryl group of rhodanese is particularly sensitive to oxidation by hydrogen peroxide or by radicals giving rise to an intramolecular disulphide bond in the inactive enzyme.[8] On the light of these considerations we reinvestigated the inactivation of rhodanese by sulphite to check the involvement in the reaction of products of sulphite autoxidation. A preliminary report of this study was already presented.[9]

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MATERIALS AND METHODS

Chemicals were of reagent grade. Sodium sulphite was from Merck. S-labelled sodium sulphite was purchased from the Radiochemical Center, Amersham. Iodoacetic acid was recrystallized from carbon tetrachloride. Crystalline beef liver catalase was from Boehringer (Mannheim). Superoxide dismutase from bovine erythrocytes was kindly supplied by Dr. G. Rotilio. Rhodanese was purified from bovine liver according to Horowitz and De Toma,[10] crystallized twice and stored at 4°C as a microcrystalline suspension in 1.8 M ammonium sulphate, 1 mM thiosulphate, pH 7.8. Proteins concentration and catalytic activity were determinated according to Sorbo.[11] Rhodanese in the sulphur-containing form, with a specific activity of about 600 units/mg, was dissolved in standard phosphate buffer (50 mM sodium phosphate, pH 7.6), and extensively dialyzed against the same buffer. Sulphur-free rhodanese was obtained by adding a stoichiometric amount of cyanide to the sulphur-containing form. The enzyme (20–40 μM, either in the sulphur-substituted or in the sulphur-free forms) was then incubated at 20°C with sodium sulphite (1–100 equiv.) in standard phosphate buffer and the reaction time courses were followed by withdrawing at time intervals aliquots of the incubation mixtures which were assayed for enzymatic activity. Rhodanese inactivation by sulphite formed by the enzymic reaction was obtained by incubating the enzyme (30 μM) with cyanide (120 equiv.) and thiosulphate (100 equiv.) in 50 mM phosphate buffer pH 7.6 at 20°C. Rhodanese derivatives were also obtained by incubating the sulphur-free enzyme with an equimolar amount of hydrogen peroxide as previously reported[8] or with a small molar excess (5 equiv.) of iodoacetate in standard phosphate buffer. To eliminate excess of reagents, the incubation mixtures were gel-filtered on a Shephadex G-25 column equilibrated with the same buffer.

Fluorescence measurements were performed using a Perkin-Elmer MPF3 spectrofluorometer. Spectrophotometric measurements were carried out with a Beckman DU-8 or 5260 spectrophotometer equipped with a temperature control unit. In samples treated with $^{35}SO_3^{2-}$ incorporation of radioactivity was determined with a Packard Tri-Carb Model 300 liquid scintillation spectrometer. The sulphydryl content of rhodanese was determined by titration with 5-5′-dithiobis-(2-nitrobenzoic acid) in the presence of 8 M urea.[12] When required, oxygen was removed from the reaction medium in a thumberg cuvette and replaced by nitrogen as previously described.[8] Electron paramagnetic resonance (EPR) experiments were carried out at room temperature with a Varian V 4502-14 spectrometer equipped with a 100-Kcycles

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field modulation unit. Sulphur trioxide anion radical was detected with 5,5-dimethyl-1-pyrrolyne-N-oxide (DMPO) as spin trap.[13]

RESULTS AND DISCUSSION

The effect of sulphite addition on rhodanese activity is shown in Figure 1A and B. An excess of reagent, at least ten times above the enzyme concentration, and the presence of oxygen in the reaction medium are absolute requirements for complete enzyme inactivation. These findings suggest that the true inactivating agent could be a product of sulphite autoxidation, rather than the starting reagent. The inactivation process is effectively antagonized by chelating traces of metal ions, that catalyze sulphite autoxidation, or by scavenging free radicals with mannitol (Figure 2). Also sodium benzoate (50 mM) has been found to prevent enzyme inactivation. Since the presence of superoxide dismutase and catalase protects the enzyme only at a small extent, the inactivation process may be mainly attributed to sulphite radicals rather than to intermediates of oxygen reduction, such as superoxide and/or hydrogen peroxide, that were previously identified as rhodanese inactivating agents.[8,14] This hypothesis is supported by detection of the sulphur trioxide anion radical in the incubation mixture under inactivation conditions. The removal of this reactive radical as DMPO–SO_3^- adduct protects enzyme against inactivation.

The kinetics of rhodanese inactivation by sulphite produced enzymatically in a reaction medium containing thiosulphate and cyanide is indistinguishable from that obtained in the presence of sulphite (data not shown). The addition of an excess of thiosulphate with respect to the sulphite concentration, in order to convert the enzyme into the less reactive sulphur-containing form, prevents the loss of enzyme activity

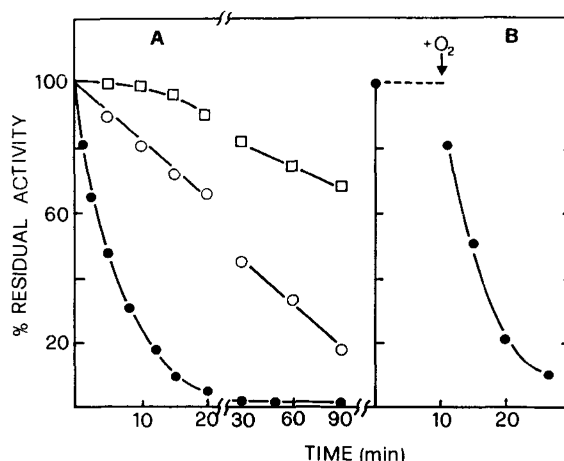

FIGURE 1 Time course of rhodanese inactivation by sulphite. A: Enzyme (30 μM) was incubated with sulphite at various molar ratios 1 : 1 (□), 1 : 10 (○), 1 : 100 (●), in 50 mM phosphate buffer, pH 7.6. B: The reaction was carried out under anaerobic conditions with enzyme and sulphite in a molar ratio 1 : 100; the arrow indicates the exposure to the air of the reaction mixture. At the indicated time aliquots were drawn from the incubation mixture and tested for activity.

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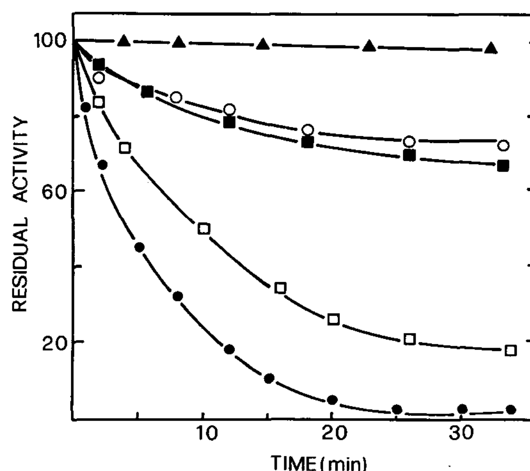

FIGURE 2 Protective effect of various compounds on rhodanese inactivation by sulphite. 30 μM enzyme was incubated with 3 mM sulphite in 50 mM phosphate buffer, pH 7.6 in the absence (●) or in the presence of the following compounds: 6 mM thiosulphate (▲), 2 mM EDTA (○), 80 mM D-mannitol (■), 0.3 mM superoxide dismutase and 50 nM catalase (□).

(Figure 2 and Table I). In agreement with this finding, when the loss of enzyme activity is caused by sulphite produced enzymatically, in the presence of thiosulphate and cyanide, an excess of the latter reagent is required to inactivate the enzyme, while cyanide alone is uneffective. The observation that the enzyme must be in the sulphur-free form to be inactivated and the finding that 1-SH group per rhodanese molecule is concomitantly lost (Table I), indicate that a specific modification of the essential sulphydryl in the inactivation process occurs.

Enzyme inactivation induced by sulphite is accompanied by spectral changes, as shown in Figure 3. Upon reaction with sulphite, the removal of transferable sulphur from the sulphur-loaded rhodanese causes an instantaneous 25% enhancement of the intrinsic protein fluoresence.[15] Subsequently, fluoroescence and absorbance changes

TABLE I

Sulphydryl groups and ^{35}S content of rhodanese before and after treatment with labelled sulphite. Enzyme (20–40 μM) was incubated with 100 molar excess of ^{35}S-labelled sulphite in 50 mM phosphate buffer pH 7.6 for 60 min at 20°C. Excess of reagents was removed by gel filtration before testing the enzyme derivative

Enzyme form	-SH group/mol	^{35}S bound/mol
native	3.6	–
native + $^{35}SO_3^{2-}$	2.8	0.8 ± 0.1
native + thiosulphate + $^{35}SO_3^{2-}$	3.5	0.1
carboxymethylated	2.8	–
carboxymethylated + $^{35}SO_3^{2-}$	2.8	0.05
oxidized by H_2O_2	2.0	–
oxidized by H_2O_2 + $^{35}SO_3^{2-}$	2.8	0.8 ± 0.1
inactivated by sulphite, then restored by Et-SH + $S_2O_3^{2-}$	3.5	0.1

All values are an average of four experiments

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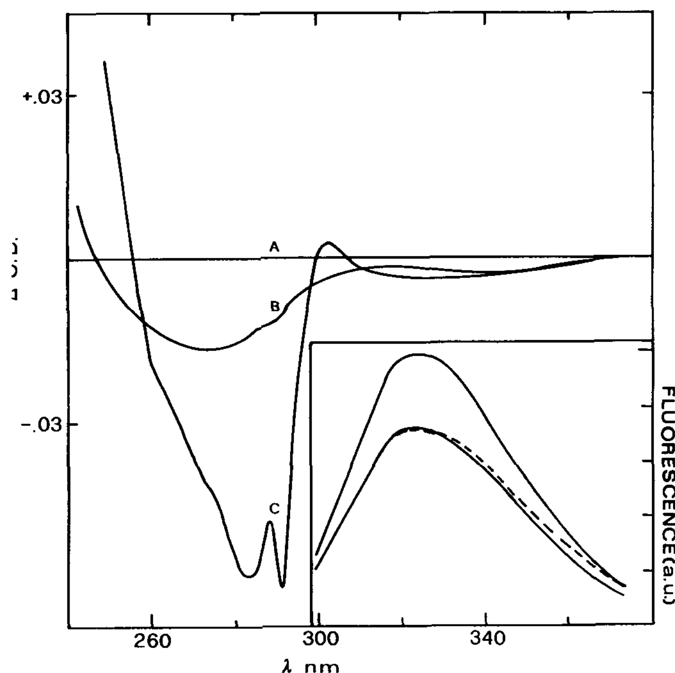

FIGURE 3 Spectroscopic changes induced by sulphite on rhodanese. Difference absorption spectra were obtained using a Beckman 5260 spectrophotometer with split cells with two 0.439 cm compartments in tandem. Curve A = base line: both sample and reference contained 53 μM sulphur-loaded enzyme (ES), in 50 mM phosphate buffer pH 7.6 separated from an equal volume of buffer. Curve B = E vs. ES: upon addition of 1 mM sulphite to the enzyme compartment of the sample and to the buffer compartment of the reference. Curve C = E-SO_3^- vs. ES: spectrum was taken 40 min after further addition of sulphite, as in B, to a final concentration of 10 mM. Reagents were added in microliter amounts and a corresponding amount of buffer was added in other compartments of sample and reference. Fluorescence spectra were obtained using a Perkin-Elmer MPF3 spectrofluorometer with 1-cm light-path cell. Curves A′, B′, and C′ correspond to the samples A, B and C described above upon 1 : 10 dilution with buffer. Excitation wavelength was 280 nm; excitation and emission slit was 10 nm. All spectra were monitored at 20°C.

concomitant with the enzyme inactivation time course become apparent: a quenching of the protein fluorescence to the level of the sulphur-loaded fluorescence intensity, a small bathochromic shift in the fluorescence emission, and an absordance perturbation in the near UV. These spectral changes are similar to those caused by other inactivating agents, such as iodoacetate and hydrogen peroxide.[16] The chemical modification of the active site cysteine by such reagents appears to cause a displacement of some tryptophyl side chains from hydrophobic to hydrophilic environment.

MECHANISM OF INACTIVATION

To further investigate the mechanism of *in vitro* inactivation of rhodanese, the incorporation of radioactivity by enzyme upon treatment with $^{35}SO_3^{2-}$ was also tested. As indicated in Table I the sulphur-free enzyme incorporates almost 1 equivalent of

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$^{35}SO_3^{2-}$ and concomitantly loses 1 -SH group, while the carboxymethylated and the sulphur-containing enzymes are unaffected by the treatment with $^{35}SO_3^{2-}$. This finding confirms that cysteinyl side-chain participates in the inactivation process.

It has been previously observed that hydrogen peroxide oxidizes the enzyme by forming an intramolecular disulphide bridge between cys-247 and a neighbouring cysteine residue.[8,17] Both the enzyme inactivated by sulphite and by hydrogen peroxide have the ability of incorporate about 1 equivalent of labelled S/mol (Table I). These findings lead us to suggest the following oxidative events caused by sulphite. Free radicals, produced by its autoxidation, oxidize the enzyme promoting the formation of an intramolecular disulphide bridge, a process that possibly involves as intermediate a sulphenyl group.[16] The enzyme containing the disulphide bond cannot be isolated because it rapidly undergoes sulphitolysis, in the presence of the excess of sulphite, and 5-sulphonate group is thus formed at the active site (Scheme 1). This hypothesis is strengthened by the observation that mercaptoethanol, which is known to cleave the S-sulphonate group,[19,20] is effective in both restoring enzyme activity and removing bound $-SO_3^-$ from the enzyme inactivated by sulphite (Table I). The concomitant presence of thiosulphate, that possibly stabilizes the enzyme by converting

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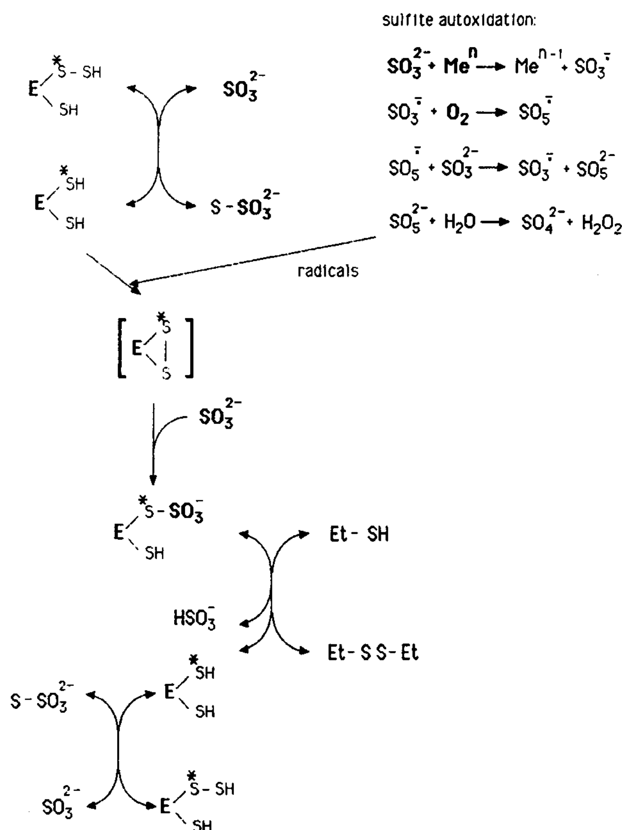

SCHEME 1 The starred sulphur is that responsible for enzyme activity.

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it into the sulphur-loaded form as soon as the -SO_3^- group has been removed, is also required in order to fully recover the enzyme activity.

The reversibility of chemical modification produced on rhodanese active site by sulphite radicals or by hydrogen peroxide[8] suggests that these processes may be of physiological importance in detoxication of oxidizing agents produced inside mitochondria.

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