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SELECTIVE REACTIVITY OF RHODANESE SULFHYDRYL GROUPS WITH 5,5'-DITHIO-BIS(2-NITROBENZOIC ACID)

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Summary

The reactivity of the sulfhydryl groups of sulfur-containing and sulfur-free rhodanese (thiosulfate : cyanide sulfurtransferase, EC 2.8.1.1) with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) has been investigated.

Only 0.6 sulfhydryl group of the sulfur-containing enzyme reacts with DTNB. After removal of sulfur from persulfide groups a further 0.6 sulfhydryl group (i.e. a total of 1.2) becomes accessible to the reagent.

The resulting enzyme-thionitrobenzoate complex shows an absorption spectrum with a shoulder at 325 nm due to bound thionitrobenzoate.

Both thiosulfate and cyanide remove thionitrobenzoate from the enzyme restoring its catalytic properties.

The modified enzyme is protected from alkylation by iodoacetate until thionitrobenzoate is removed.

The existence of a further sulfhydryl group close to the catalytic one is suggested.

Introduction

The importance of sulfhydryl groups in the reaction catalyzed by rhodanese (thiosulfate : cyanide sulfurtransferase, EC 2.8.1.1) has been pointed out by several authors [1—3]. The enzyme contains 4 sulfhydryl groups per molecule of molecular weight 37 000, [4] and their functional role has been explained on the supposition that rhodanese is formed by two identical subunits [5].

Recent studies on the molecular weight of rhodanese report that the enzyme consists of a single polypeptide chain [6—9]. X-ray crystallographic study of rhodanese shows that the molecule is composed of two separate and very similar globular regions connected by a long loop [10]. The same authors reported that a dimeric molecule can result from proteolytic attack on the loop

connecting the two portions of originally monomeric rhodanese molecule.

In particular it has been demonstrated that one of the two cysteine residues present in each globular region is involved in the sulfur transfer reaction through the formation of a persulfide group [11].

The enzyme may exists in a sulfur-containing and in a sulfur-free form [12]. Carboxymethylation of the sulfur-free enzyme has shown that alkylation is concomitant with loss of activity [3]. The second pair of cysteine residues on the other hand is not accessible to external agents i.e. it is resistant to alkylation and has been considered as non-essential for activity [2,5].

Studies with the most widely used reagents for determining sulfhydryl groups have shown that the enzyme contains no disulfide. The four sulfhydryl groups present in the molecule are accessible only in denaturing conditions [1, 13,14].

Since rhodanese contains these two different types of sulfhydryl groups it seemed interesting to investigate whether it was possible to obtain a derivative of sulfhydryl groups of rhodanese which may evidentiate a different reactivity of these groups. In this paper we describe some properties of a rhodanese-thionitrobenzoate mixed disulfide.

Materials and Methods

Chemicals were from Merck, Fluka and Sigma, of reagent grade. Urea was recrystallized twice from methanol. $K^{14}CN$ was purchased from the Radiochemical Centre, Amersham.

Crystalline rhodanese was purified from bovine liver as described by Horowitz and DeToma [15]. The enzyme with a specific activity of 600 units/mg was dissolved in 50 mM phosphate buffer, pH 8, and dialyzed overnight against the same buffer. Rhodanese in the sulfur-free form was prepared as previously described [16]. Protein concentration was determined from the absorbance at 280 nm using a molar extinction coefficient $\epsilon = 64.75 \cdot 10^3$ and a molecular weight of 37 000 [17].

The reaction of DTNB with rhodanese both in the sulfur-containing and in the sulfur-free form was followed at 412 nm upon the addition of DTNB vs. appropriate blank containing all the reagents without protein. The molar ratio between enzyme and DTNB was 1 : 25. Absorbance readings were done with a Beckman DU2 spectrophotometer. The extinction coefficient of thionitrobenzoate at 412 nm was taken to be 13 600 $M^{-1} \cdot cm^{-1}$. When no further increase in absorbance at 412 nm was observed the sample was filtered through a column (1 × 30 cm) of Sephadex G-25 in 50 mM phosphate buffer, pH 8, in order to separate the enzyme-thionitrobenzoate complex from the excess of reagent and product. The rhodanese-thionitrobenzoate complex was stable for at least 48 h if stored in the cold. The cleavage of the mixed disulfide between enzyme and thionitrobenzoate was followed spectrophotometrically after addition of 2-mercaptoethanol, cyanide or thiosulfate. Absorption spectra in the 250—500 nm region were monitored with a Beckman Acta III spectrophotometer equipped with a temperature control unit. When the enzyme-thionitrobenzoate complex was cleaved with ^{14}C-labeled cyanide the reaction products were separated by gel filtration on a Sephadex G-25 column (1 × 30 cm). Then the radio-

activity of the protein was measured with a Packard Tri-Carb Model 3380 liquid scintillation spectrometer.

Quantitative determination of sulfhydryl groups was performed according to the method of Ellman [18] in the presence of 8 M urea as denaturing agent. In this case all the free sulfhydryl groups react only if DTNB was added to the protein sample before urea addition. This behaviour is in agreement with a recent report by Baillie and Horowitz [14].

Alkylation experiments were performed at 20° C incubating the enzyme-thionitrobenzoate complex or the sulfur-free rhodanese with 15 molar excess of iodoacetate in 50 mM phosphate buffer, pH 8. Aliquots were taken from the reaction mixtures at appropriate time and activity was tested in the standard assay [17] but after 1 min incubation.

Results and Discussion

Reaction of rhodanese with DTNB

When the enzyme is as purified (i.e. sulfur-containing enzyme) the sulfhydryl groups of 27 μM rhodanese react poorly with 0.75 mM DTNB at pH 8 and 23° C. Fig. 1 shows that the extent of reaction is temperature dependent and is higher when the substrate sulfur is removed from the enzyme. No experiment can be made beyond 30° C as the protein undergoes denaturation. The data reported indicate that 1.2 mol of sulfhydryl groups/mol enzyme are titrated when rhodanese in the sulfur-free form is incubated at 30° C with 25 molar excess of DTNB.

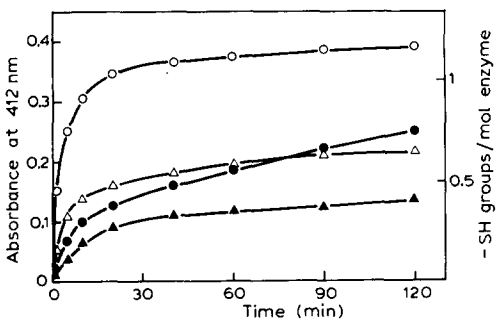


Fig. 1. Reaction of rhodanese with 5'-dithio-bis(2-nitrobenzoic acid). 27 μM rhodanese in 50 mM phosphate buffer, pH 8, was incubated with 0.75 mM DTNB at 23°C (Δ) or 30°C (○). Solid symbols: sulfur-containing enzyme, open symbols: sulfur-free enzyme.

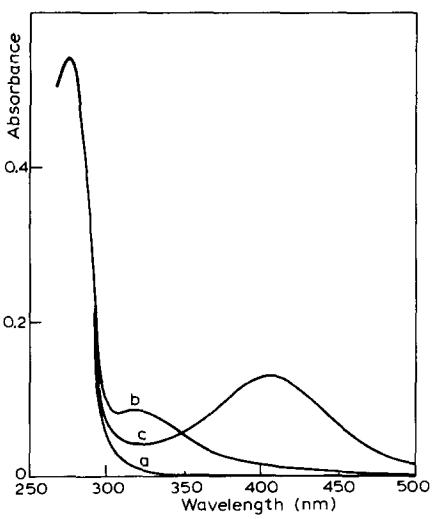


Fig. 2. Absorption spectra of rhodanese. Sulfur-free rhodanese (8.4 μM) in 50 mM phosphate buffer, pH 8, as prepared (curve a); after 1-h incubation with 0.21 mM DTNB at 30°C followed by gel filtration (curve b); after reduction of the enzyme-thionitrobenzoate complex with 0.62 mM 2-mercaptoethanol (curve c).

It is possible to suppose that sulfhydryl groups which participate in the enzymatic reaction are protected from the reaction with DTNB by transferable sulfur bound as a persulfide group [11]. The amount of sulfhydryl groups determined with DTNB on the sulfur-free enzyme is lower than would be expected by the possible occurrence of two active sites. Determinations of substrate sulfur bound to the enzyme give, with different methods [19,20], an average of 1.35 sulfur atoms/molecule enzyme; as this number is close to that for DTNB reaction it is reasonable to suppose that for both reactions less than two essential sulfhydryl groups are accessible.

These results can be also explained in view of the conformation changes that rhodanese undergoes when the enzyme is in the sulfur-containing or in the sulfur-free form. As reported by Volini and Wang [21] the native protein is in a conformation upon sulfur release via the transition from a β-type structure to a disordered coil-type structure involving movement of a particular group of aminoacid residues. The presence of an ordered structure in the sulfur-containing enzyme is in agreement with our hypothesis of the need of a particular folding to stabilize the persulfide group which is accessible only to acceptor substrates [11]. Similarly the conditions for reaction of several compounds like DTNB or iodoacetate with the essential sulfhydryl groups can be correlated to a disordered structure in the sulfur-free enzyme.

The assay of activity during the reaction of the sulfur-free enzyme with DTNB shows no differences between the modified enzyme and a control untreated enzyme. These results apparently eliminate the possibility that the modified sulfhydryl groups could be those required for catalytic activity. However the enzyme might be active because the thionitrobenzoate group is removed from the protein during the assay by the very large excess of cyanide (see below).

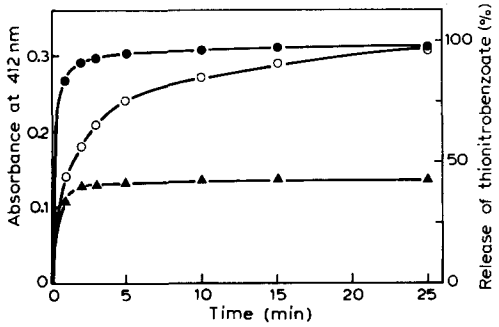
When solid urea (8 M final concentration) was added to the incubation mixture all the four sulfhydryl groups react in few minutes with DTNB, whatever form of enzyme was used. Thus only by denaturation of protein it is possible to expose the unreactive thiol groups.

Characterization of the rhodanese-thionitrobenzoate complex

In order to investigate the identity of sulfhydryl groups which react with DTNB when the enzyme is in the sulfur-free form, the protein-thionitrobenzoate adduct was separated from excess reagent by gel filtration on a Sephadex G-25 column in 50 mM phosphate buffer, pH 8.

The absorption spectrum of the rhodanese-thionitrobenzoate complex shows the presence of an absorption in the form of a shoulder at 325 nm (Fig. 2b) while the untreated enzyme in the sulfur-free form does not show any definite absorption in this region (Fig. 2a). This absorption at 325 nm has an ϵ of 8 000 $M^{-1} \cdot cm^{-1}$ and may be ascribed to thionitrobenzoate bound to rhodanese thiol groups. The addition of a reducing agent like 2-mercaptoethanol bleaches the absorption at 325 nm with a concomitant appearance of a peak at 412 nm (Fig. 2c) due to the colored anion released. Spectrophotometric determination of thionitrobenzoate released under these conditions gives 1.2 mol/mol enzyme.

Substrates are also able to remove thionitrobenzoate bound to the enzyme. In Fig. 3 are reported the rates of enzyme-thionitrobenzoate reaction with cyanide or with thiosulfate in comparison to that observed with 2-mercapto-



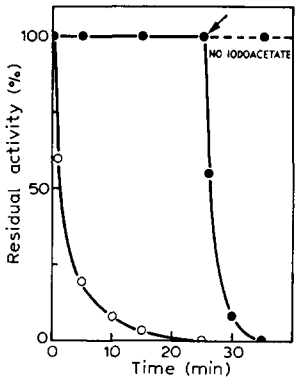
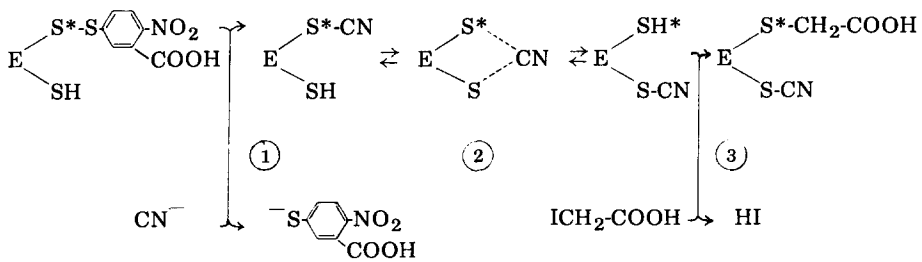


Fig. 3. Time course of rhodanese-thionitrobenzoate complex cleavage. The experiments were carried out incubating 23 μM enzyme-thionitrobenzoate complex, in 50 mM phosphate buffer pH 8, with 1.75 mM 2-mercaptoethanol (•), with 1.75 mM cyanide (○) or with 20 mM thiosulfate (▲).

Fig. 4. Effect of thionitrobenzoate on rhodanese inactivation by iodoacetate. Rhodanese-thionitrobenzoate complex (23 μM in 50 mM phosphate buffer pH 8) was incubated with 15 molar excess of iodoacetate (•). The arrow indicates the addition of cyanide to a final concentration of 1.75 mM; the dotted line represents the same experiment in the absence of iodoacetate. Open circles: control of inactivation of sulfur-free enzyme in the same condition.

ethanol. At a concentration of thiosulfate 10 time higher than cyanide only the 45% of thionitrobenzoate is released while the remaining amount is bound to the enzyme as determined by the absorption at 325 nm. This different behaviour of the two substrates in the cleavage of the mixed disulfide present in the enzyme-thionitrobenzoate complex is due to their different thiophilic strength These data show that the mixed disulfide present in the rhodanesee-thionitrobenzoate molecule is cleaved by substrates in a preferential way: the leaving thionitrobenzoate is never bound to substrates which instead bind to the protein sulfhydryl groups as indicated for cyanide in step 1 of scheme 1. To check this a sample of rhodanese-thionitrobenzoate complex was cyanolyzed with 75



Scheme 1. The starred sulfur is that responsible for enzyme activity.

molar excess of $^{14}CN^-$. The mixture was then sieved on a Sephadex G-25 column in 50 mM phosphate buffer, pH 8. Measures of radioactivity of protein give 0.8 mol of cyanide/mol enzyme. This result agrees with a more detailed study on the cleavage of unsymmetrical disulfide by Parker and Kharasch [22].

A further experiment shows that no radioactivity was recovered in the pro-

tein when the persulfide group present in the enzyme was cyanolized in the standard procedure used to prepare sulfur-free enzyme. This means that in a peculiar unsymmetrical disulfide, like the persulfide group, the outer sulfur atom undergoes as the nucleophilic attack by cyanide.

The enzyme-thionitrobenzoate complex retains the sulfur-transferring ability in the standard assay method. This result could be explained in two different ways: either the sulfhydryl groups of rhodanese bound to thionitrobenzoate are not important for activity or the adduct is cleaved by cyanide present in the assay mixture. The latter possibility appears to be supported by the above-reported data. However the resulting cyanide subsituted enzyme (see Scheme 1) should be inactive. This is in contrast with the finding that also rhodanese-thiocyano derivative retains its activity. To reconcile these findings it should be pointed out that a compound containing a free thiol group is able to remove cyanide bound to a protein cysteinyl residue [23]. In our condition it is possible to suppose that cyanide can be shifted to the other sulfhydryl group thus restoring the activity (step 2 of scheme 1).

A further check to establish the binding site of thionitrobenzoate is obtained by treating rhodanese-thionitrobenzoate complex with iodoacetate. Rhodanese in the sulfur-free form is rapidly inactivated by iodoacetate while it is unaffected in the sulfur-containing form [2,3]. This effect may be attributed to a protection of the essential thiol group exerted by bound sulfur. As reported in Fig. 4 the activity of the enzyme-thionitrobenzoate complex is unaffected by iodoacetate while under the same conditions the sulfur-free enzyme is rapidly and completely inactivated. The enzyme-thionitrobenzoate complex becomes sensitive to iodoacetate only after addition of cyanide to the incubation mixture.

As expected a similar inactivation is obtained if the enzyme-thionitrobenzoate complex is first cyanolyzed with 75 molar excess of labeled cyanide and then treated with iodoacetate in the same experimental conditions. In this way, as reported in step 3 of Scheme 1, all the sulfhydryl groups of the enzyme are modified: the catalytic sulfhydryl groups alkylated by iodoacetate and the remaining sulfhydryl groups labeled by cyanide.

We hope to use this enzyme-thiocyano derivative for the study of specific chemical cleavage of the rhodanese polypeptide chain at cysteine residue as reported by Vanaman and Stark [24].

The experiments reported above lead us to conclude that sulfhydryl groups of rhodanese when the enzyme is in the sulfur-containing form are not accessible to DTNB. In the presence of a denaturing agent a very rapid reaction is obtained with four sulfhydryl groups per rhodanese molecule. The sulfur-free undenaturated enzyme shows higher reactivity with DTNB without modification of the catalytic properties. Since in these conditions the amount of thionitrobenzoate bound to the enzyme coincides with the amount of persulfide that was in the native enzyme, it is possible to suppose that in the sulfur-free enzyme the sulfhydryl groups which bind substrate sulfur become accessible to the reagent. This hypothesis is confirmed by the properties of the enzyme-thionitrobenzoate derivative. The involvement of the essential sulfhydryl group in the mixed disulfide with thionitrobenzoate explains why thionitrobenzoate must be removed from the adduct before the essential sulfhydryl groups can be

alkylated. The proximity of a second sulfhydryl group to the catalytic one may be assumed to keep active the rhodanese-thiocyano derivative by shifting the bound cyanide from the active site. This assumption is supported by recent reports [14,25,26] that, under certain circumstances, the essential sulfhydryl group may react with a second sulfhydryl group close to it to give a disulfide bond.

Very recently Ploegman [27] reported the three-dimensional structure of bovine liver rhodanese. The stereopicture of the active site region of the enzyme shows two sulfhydryl groups close enough to explain the interaction postulated above.

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