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PROBING THE ACTIVE SITE OF RHODANESE WITH DISULFIDE REAGENTS

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Abstract

The purpose of the present investigation was to obtain an insight into the chemical properties of the active site of the enzyme rhodanese (thiosulfate: cyanide sulfurtransferase). This enzyme is widely distributed in nature, from bacteria to man. Several functions, including cyanide detoxification and synthesis of iron-sulfur centers, have been proposed for this enzyme. Different disulfides with different chemical properties were allowed to react with the sulfhydryl group in the active site of rhodanese. The Ellman reagent; 5,5'-dithiobis (2-nitrobenzoic acid), DTNB, reacted very slowly. A marked increase in the rate of reaction of rhodanese with DTNB was observed when cationic disulfides, cystamine and cystine, were present. No change was seen with non-cationic disulfides dimethyl disulfide, 2-hydroxy ethyl disulfide, dithioglycolic acid oxidized glutathione, 2,2'-salicylic acid, 2,2'dipyridyl disulfide (2-PDS) and 4,4-dipyridyl disulfide (4-PDS). A Km of 2.0 and 2.5 mmol/l was obtained for cystamine and cystine, respectively, while the Km for thiosulfate, the common substrate of the enzyme was 3.0 mmol/l. These results possibly indicate the existence of anionic groups in or in the vicinity of the active site which facilitate binding of the cationic disulfides. Furthermore, these data might suggest that these disulfides are the true physiological substrates of rhodanese.

Introduction

Rhodanese (E.C.2.8.1.1.; thiosulfate:cyanide sulfurtransferase) is a ubiquitous enzyme present in all living organisms from bacteria to man [1-11]. Several biological functions, including cyanide detoxification [13-15], construction of iron-sulfur centers in proteins such as ferredoxin [17], succinate dehydrogenase [17], and NADH dehydrogenase [18], and maintenance of the sulfane sulfur pool in living organisms [19], have been suggested for this enzyme.

Rhodanese catalyzes sulfur transfer between thiosulfate and cyanide with release of sulfite and thiocyanate. The reaction occurs by a double displacement mechanism with the formation of an intermediate enzyme-sulfur complex [12, 19]:

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

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

In the first half reaction, the sulfur-free enzyme (ES^-) binds reversibly the outer sulfur of thiosulfate on the enzyme as a persulfide group ($ES\text{-}S^-$) releasing sulfite. In the second half reaction, the enzyme trans-

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fers this sulfur to cyanide to produce thiocyanate.

Rhodanese has been crystallized [20], sequenced and its three dimensional structure determined by X-ray crystallography [21]. The enzyme is a single polypeptide chain containing 293 amino acids, four cysteine residues with no disulfide bond [22, 23]. The sulfhydryl group of cysteine-247 has been identified as the site of covalent attachment of sulfane sulfur in sulfur-rhodanese [22]. Crystallographic and chemical modification studies have shown that side chains of phenylalanine-212, phenylalanine-106, tyrosine-107, tryptophan-35, and valine-251 provide the hydrophobic wall of the active site and the guanidino group and the ε-NH_2 group of arginine-186 and lysine-249, respectively, are positioned in a way to suggest possible participation in binding of anionic substrate [21, 24].

In the present investigation, it was attempted to further explore the chemical properties of the sulfhydryl group in the active site by employing the sulfhydryl-disulfide system originally defined by Wilson *et al.* [25]. The evidence obtained in this study suggests the possible involvement of anionic groups of the side chains of amino acids of the enzyme molecule in binding cationic disulfides. These results have implications with respect to the mechanism of thiol-disulfide exchange reactions catalyzed by rhodanese and might define a new physiological function for rhodanese.

Materials and Methods

Materials

Sodium thiosulfate, potassium cyanide, and potassium thiocyanate were from Merck (Darmstadt, FRG). 5,5'-Dithiobis (2-nitrobenzoic acid), (DTNB), cystamine hydrochloride, cystine hydrochloride, 2,2'-salicylic acid, 2,2'-dipyridyl disulfide (2-PDS) and 4,4-dipyridyl disulfide (4-PDS), were from Sigma Chemical Co. (Saint Louis, Mo, USA). Dimethyl disulfide, 2-hydroxy ethyl disulfide and dithioglycolic acid were from Aldrich Chemical Co. (Milwauki, Wi, USA) and oxidized glutathione was from Fluka Chemie AG, Switzerland. All other chemicals were of analytical grade and were obtained from commercial sources.

Methods

A. Purification of Rhodanese

Bovine liver rhodanese was isolated and purified using several steps of ammonium sulfate and isoelectric precipitation as described by Horowitz and Detoma [26]. The enzyme was purified to a constant specific activity of 70 units/mg protein and stored at -20°C in ammonium sulfate as crystalline suspension of the sulfur containing (ES-S^-) form.

B. Assaying Rhodanese Activity

Rhodanese activity was assayed by a colorimetric method based on the absorption at 460 nm of the ferric-thiocyanate complex formed with the reaction product thiocyanate [20]. The reaction mixture contained 16.8 mmol/l sodium thiosulfate, 40 mmol/l glycine buffer pH 9.2, 16.7 mmol/l KCN and 30 µl of enzyme solution in a final volume of 4.0 ml. The reaction was carried out for 20 min at 37°C and stopped by adding 0.5 ml 38% formaldehyde. Formaldehyde was added to control tubes prior to the addition of enzyme solution. The concentration of thiocyanate was determined as follows [20]: Samples were mixed with one ml of ferric nitrate solution containing 0.025 g $Fe(NO_3)_3 9\ H_2O$ in 0.74 ml water and 0.26 ml concentrated nitric acid. After centrifugation of the mixture to remove the interfering turbidity, the absorbance was measured at 460 nm and the concentration of thiocyanate formed was obtained from standard curve which was produced by treating solutions containing different concentrations of thiocyanate as described above. In this study, pH 9.2 was used instead of pH 7.4 (which is routinely used) in order to decrease the turbidity of solutions after addition of ferric nitrate. No significant difference was observed when purified rhodanese was assayed at pH 7.4 or 9.2. The unit of enzyme activity was defined as micromoles thiocyanate formed per min at 37°C and pH 9.2. In order to obtain kinetic data, the assaying of rhodanese was performed at different concentrations of one of the substrates (thiosulfate or cyanide) while keeping the concentration of the other constant.

C. Preparation of Sulfur-Free Rhodanese

The sulfur-free rhodanese (E^- form) was obtained by adding 10-fold molar excess of potassium cyanide to a rhodanese solution [27] and dialyzing against 0.1 mol/l sodium phosphate buffer pH 8.0 to remove excess of cyanide.

D. Determination of Protein Concentration

Protein concentration was determined using an extinction coefficient at 280 nm of 17.5 for a 1.0% solution of rhodanese [20].

E. Blocking Sulfhydryl Group of Rhodanese

The sulfhydryl group of rhodanese was blocked by its reaction with iodoacetate as described by Wang and Volini [28]. Briefly, to 1.0 ml of 0.1 mol/l glycine buffer, pH 9.1 containing 0.1 mmol/l iodoacetate was added 0.1 ml of a 125 mg/ml solution of rhodanese and the mixture was incubated at 25°C for one hour. The mixture was then dialyzed against one liter of sodium phosphate buffer, pH 8.0 to remove excess of iodoacetate. A control rhodanese sample which went through all steps in the absence of iodoacetate was

also prepared. These samples were used in subsequent studies.

F. Reaction of Rhodanese with DTNB

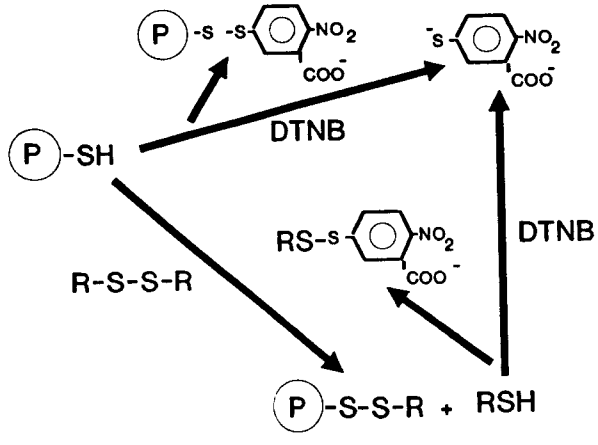
Proteins react with DTNB and liberate one mole thionitrobenzoate (TNB) per mole SH group of protein. From the molar extinction coefficient of TNB (which is 13600 M^{-1} Cm^{-1}) one can calculate the number of moles of available SH groups of proteins [29]. All experiments described here and in the following sections were performed on the sulfur-free rhodanese (E^-) and on the iodoacetate modified rhodanese. The reaction mixture contained 5 mmol/l DTNB in 0.95 ml of 0.1 mol/l sodium phosphate buffer pH 8.0. The reaction was started by adding 50 µl enzyme solution containing 1.7 mg rhodanese and absorbance at 412 nm was recorded against a blank (which contained all reagents except that 50 µl water was used instead of rhodanese) using a Unicam model SP 1800 recording spectrophotometer (Pye Unicam Ltd, Cambridge, UK). After the reaction was complete, the solution was dialyzed against two liters of 0.1 mol/l sodium phosphate buffer and then used for assaying rhodanese activity. This sample was labeled rhodanese-TNB.

G. Determination of Total Sulfhydryls

Total sulfhydryl of rhodanese was determined as above except that the reaction mixture contained 8.0 mol/l urea.

H. Reaction of Rhodanese with DTNB in the Presence of Other Disulfides

A modified procedure of Wilson *et al.* [25] was used to follow the reaction of disulfides which do not produce a chromogen when reacting with sulfhydryl groups [25] (Scheme I). The reaction was done as described in the preceding section (section F) except that the reaction mixture contained 2.5 mmol/l disulfide reagent. Kinetic studies to assess the catalytic effect of rhodanese on disulfide reagents were performed using the reaction of rhodanese with DTNB in the presence of different concentrations of disulfide reagents. Initial velocities were calculated from the best straight-line fits to the initial portions of progress curves.



Scheme I. Reaction of sulfhydryl group of rhodanese with DTNB in the presence of disulfide reagents

I. Reaction of Rhodanese with 2-PDS and 4-PDS

2-PDS and 4-PDS react with sulfhydryl groups to produce 2-thiopyridine (2-TP) and 4-thiopyridine (4-TP), respectively. The amounts of sulfhydryl reacting with these reagents can be measured from the extinction coefficients which are 7.0×10^3 M^{-1} cm^{-1} and 2.0×10^4 M^{-1} cm^{-1} for 2-TP and 4-TP, respectively [30]. The methodology for 2-PDS and 4-PDS was similar to that described by Grasetti and Murray [30]. The reaction was started by adding 50 µl enzyme solution containing 1.7 mg rhodanese to 0.95 ml of 0.1 mol/l sodium phosphate buffer pH 8.0 containing 5 mmol/l 2-PDS or 4-PDS and recording absorbance at 343 or 324 nm, respectively. After the reaction was completed, the solutions were dialyzed against two liters of 0.1 mol/l sodium phosphate buffer and then used for assaying rhodanese activity. These samples were labeled rhodanese-2TP and rhodanese-4TP.

Results

DTNB alone reacted with rhodanese slowly and absorbance at 412 nm reached highest value after 40 min (Fig.1, A). The results of reaction of rhodanese with 2-PDS and 4-PDS were similar to DTNB (Fig.1, B). The absorbance at respective wavelengths

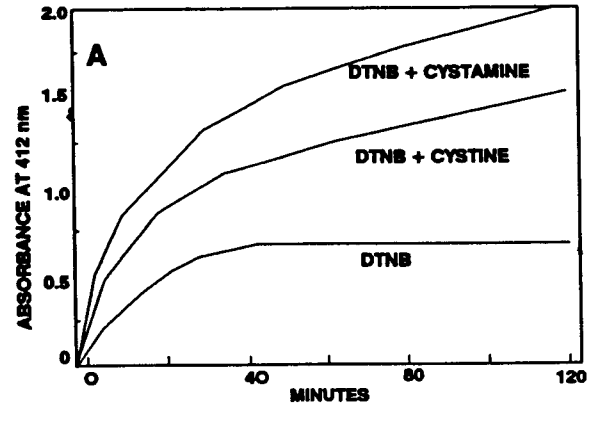


Figure 1a. Time course of reaction of rhodanese SH group with DTNB in the absence and presence of disulfides. The lowest curve represents reaction in the presence of DTNB alone or DTNB in the presence of dimethyl disulfide, 2-hydroxy ethyl disulfide dithioglycolic acid, oxidized glutathione and 2,2'-salicylic acid.

corresponds to one mole SH group per mole rhodanese if a molecular weight of 33000 Daltons is taken into account for bovine liver rhodanese [21] (Table 1). In the presence of 8 mol/l urea, the reaction was complete in less than 5 min and a total of four sulfhydryl groups were titrated with DTNB. No reaction occurred when iodoacetate-treated rhodanese was allowed to react with DTNB, indicating that the sulfhydryl group(s) in rhodanese were blocked (Table 1). Iodoacetate treated and urea denatured rhodanese did not show activity towards thiosulfate or cyanide. Both thiosulfate and cyanide liberated one mole of TNB, 2-TP, or 4-TP from one mole of rhodanese-TNB, rhodanese-2TP, or rhodanese-4TP, respectively, as evidenced by an increase in the absorbance at respective wavelengths and restoration of the enzymatic activity (Table 1).

A sharp increase in the absorbance at 412 nm was observed when cystamine or cystine were included in the DTNB assay (Fig.1, A). No change in the rate of reaction occurred when other disulfides were added to the reaction mixture of rhodanese and DTNB. When the initial velocities were plotted vs. different concentrations of cystamine or cystine, hyperbolic curves characteristic of Michaelis-Menton behavior were observed. In Figure 2, the data for cystamine are shown. From double reciprocal plots the Michaelis constant (Km) for cystamine was calculated to be 2.0 mol/l (Fig.2, B). From similar plots the Km values for cystine, thiosulfate and cyanide were obtained (Table 2).

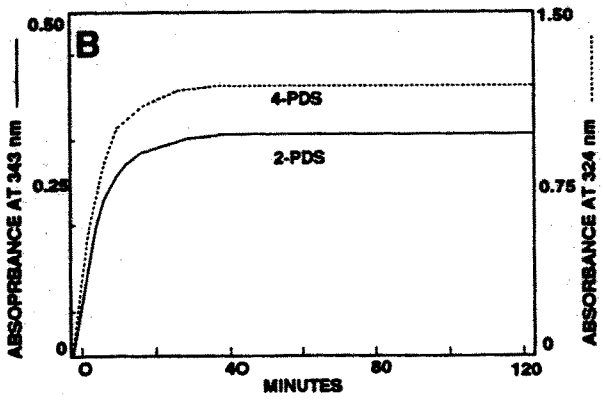


Figure 1b. Time course of reaction of SH group of rhodanese with 2,2'-dipyridyl disulfide (2-PDS) and 4,4-dipyridyl disulfide (4-PDS)

Table 1. Sulfhydryl content and activity of chemically modified rhodanese

	Mole SH per mole enzyme	% Relative activity[a]
Unmodified rhodanese	~ 1[b]	100
Rhodanese-TNB	0.98[c]	95
Rhodanese-2TP	1.08[d]	93
Rhodanese-4TP	0.97[e]	94
Iodoacetate-treated rhodanese	0.0	0.0
Urea-treated rhodanese	4.0	0.0

a: % activity relative to unmodified rhodanese
b: Obtained by reacting rhodanese with DTNB
c: Obtained by adding thiosulfate to DTNB treated rhodanese (see methods)
d: Obtained by adding thiosulfate to 2-PDS treated rhodanese (see methods)
e: Obtained by adding thiosulfate to 4-PDS treated rhodanese (see methods)

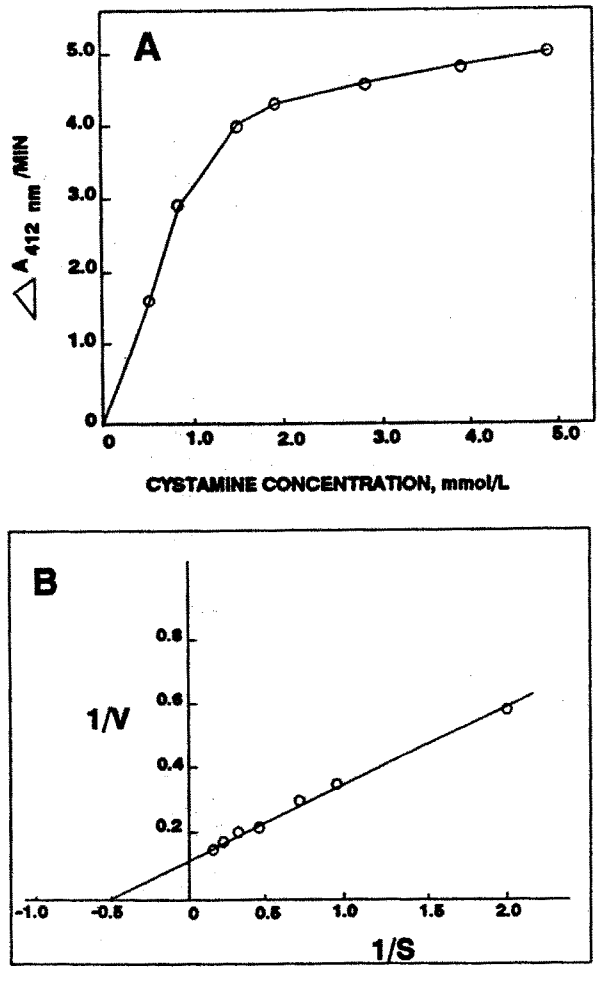


Figure 2. The effect of concentration of cystamine on the initial velocity of reaction of SH group of rhodanese

Table 2. Michaelis constants for different reagents reacting with rhodanese

Reagents	Km (mmol/l)
Thiosulfate	3.0
Cyanide	6.0
Cystine	2.5
Cystamine	2.0

Discussion

Kinetic studies of the reaction of protein nucleophiles with site-specific reagents can provide information about the local environment and its effect on the chemical reactivity, knowledge of which is essential for understanding the function of these groups in proteins [31]. Because of the importance of sulfhydryl and disulfide groups in biochemistry, substantial effort has been expended on designing specific reagents for their study and quantitation. One of the most widely used is Ellman's reagent, 5,5'-dithiobis (2-nitrobenzoic acid), DTNB. This reagent is useful because it is commercially available, water soluble, reacts with a favorable equilibrium constant with sulfhydryl groups, and especially because it generates an intensely chromophoric product, thionitrobenzoate, which can be easily monitored spectrophotometrically [25].

The data provided in this study indicated that rhodanese reacts with DTNB very slowly and after 40 min one sulfhydryl group was titrated. The thionitrobenzoate attached to the SH group could be removed by thiosulfate or cyanide, restoring the activity rhodanese (Table 1). Similar observation has been made by Pensa *et al.* [32]. This slow reactivity of SH group might be due to steric hinderance, charge repulsion, or a combination of both factors. No reaction occurred when SH group in native rhodanese was blocked by iodoacetate. When the reaction of rhodanese with DTNB was studied in the presence of 8.0 mol/l urea, which completely denatured rhodanese [32], all four SH groups of rhodanese reacted rapidly, further substantiating the notion that the negatively charged and bulky groups of DTNB might not have easy access to the active site of rhodanese. 2-PDS and 4-PDS are structurally similar to DTNB except that there is no negatively charged group on their molecule. The identical reactivity of DTNB, 2-PDS and 4-PDS towards the SH group of rhodanese (Figs. 1a and 1b) indicates that the negative charge is not involved in binding these disulfides to rhodanese.

The fact that the charge and size of DTNB, 2-PDS and 4-PDS are fixed limits the amount of information that may be obtained about the sulfhydryl group in the active site. It would be more useful to measure the rates of reaction of sulfhydryl with a variety of disulfides, RSSR, so that the influence of charge, size, or stereochemistry might be delineated [25]. Normally, however, such reactions would not produce chromophores and could only be followed with difficulty, for example, by monitoring the incorporation of labeled disulfides in the proteins. Scheme I shows that protein SH groups which react slowly with DTNB may be studied kinetically with a wide variety of nonchromogenic disulfides by a simple spectrophotometric method. A protein SH group can react with DTNB or RSSR in competition if both are present in the solution. Since the rate of reaction of RSH with DTNB is fast compared with the preceding step in which it is generated [33, 34], then the rate of appearance of thionitrobenzoate at 412 nm would be the sum of the rates of reaction of protein sulfhydryl group with DTNB and RSSR. This method allows the reactivity of various RSSR's with the SH group of rhodanese to be monitored. Among the disulfides studied, only cystamine and cystine showed enhanced reactivity towards SH of rhodanese (Fig.1 A). The amino groups of cystine and cystamine are positively charged while other disulfides are either negatively charged or are not charged at all at the pH 8.0 used in this study. Oxidized glutathione has a positively charged amino group, but it is too far from the disulfide bond and probably cannot affect the reactivity of its disulfide group with the SH of rhodanese.

The implication of the results obtained in this study are several fold. First, enhanced reactivity of cationic disulfides suggest that these disulfides provide a counter ion to the negatively charged groups close to the sulfhydryl group of rhodanese. Crystallographic studies have identified three salt bridges at the active site of rhodanese between carboxyl groups of glutamic acids-71,-193,-46 and guanidino groups of argininies-248,-186, and -41, respectively [35]. Cystamine and cystine might compete with the guanidino groups and bind to the active site in a way which allows access to the buried sulfhydryl group. Similar effects have been reported for bovine serum albumin [25] and penalbumin [36]. Second, as rhodanese has been shown to participate in the synthesis of iron-sulfur centers in proteins [16-18], the affinity of this enzyme for positively charged disulfides indicates that positively charged groups might also be present in the environment of the iron-sulfur centers. Finally, the data presented in this communication might define a new

physiological role for rhodanese. During the first twenty years of its existence as a recognized enzyme, rhodanese had only two known substrates: SSO_3^{2-} as donor of sulfur and CN^- as acceptor [19]. However, ever since, it has been recognized that rhodanese accepts many other sulfur donors such as thiosulfonates, inorganic polysulfides [12] and the trisulfide thiocystine [37]. Figure 1-A indicates that rhodanese is indeed performing a catalytic action on cystamine and cystine. The Km for cystamine and cystine is lower that that for the normal substrate, thiosulfate (Table 2), suggesting that the enzyme has a greater affinity for these cationic disulfides and that these compounds might indeed be the true physiological substrates for rhodanese. By using these disulfides, rhodanese might participate in sulfhydryl-disulfide exchange reactions, thereby maintaining a reduced state in the cells. Taken together, these data illustrate the potentiality of disulfide reagents as site-specific probes for elucidating the mechanism of action of SH groups in proteins.

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