Biochimica et Biophysica Acta, 445 (1976) 104—111

BBA 67880

REACTION OF RHODANESE WITH DITHIOTHREITOL

LAURA PECCI, BERNARDO PENSA, MARA COSTA, PIER LUIGI CIGNINI * and CARLO CANNELLA

*Institute of Biological Chemistry, University of Rome, Rome, Institute of Biochemistry, University of Parma, Parma, and * Institute of Physical Chemistry, University of Rome, Rome (Italy)*

(Received February 3rd, 1976)

Summary

The reaction between bovine rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) and reduced dithiothreitol has been studied. This reagent, in the absence of thiosulfate, reduces the amount of sulfur carried by rhodanese with formation of sulfide and oxidized dithiothreitol: E-S-SH + reduced dithiothreitol → E-SH + HS^- + oxidized dithiothreitol, (E = enzyme).

An inactivation was observed at high dithiothreitol/enzyme ratios or at very low enzyme concentrations. The inactivation was not observed in the presence of thiosulfate and can be reversed by cyanide or thiosulfate.

A thiosulfate reduction activity of rhodanese was also found using dithiothreitol as reductant.

Introduction

The sulfur transfer reaction catalyzed by rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) proceeds through a double displacement mechanism [1]. Crystalline rhodanese has been identified as an intermediate enzyme-sulfur complex [2]. Several hypotheses have been advanced to explain the chemical form of sulfur bound to the enzyme [3]. As we have previously reported [4] rhodanese shows a characteristic absorption at 335 nm which has been attributed to the presence of transferable sulfur bound in a persulfide form. The persulfide group is formed by the addition of sulfane sulfur to a cysteinyl residue essential for rhodanese activity [5].

It has been recently observed that rhodanese is inhibited by dithiothreitol, and this inhibition has been attributed to the reduction of a disulfide bond needed for the sulfur transfer reaction [6]. Since the interaction of transferable sulfur with a sulfhydryl group or with a disulfide bond seems to be debatable we have studied the reactivity of these groups with dithiothreitol. The present

paper reports the reaction of rhodanese with dithiothreitol. This reducing agent, like lipoate [7], can be utilized as substrate in the enzymic reduction of thiosulfate to sulfite and sulfide. A preliminary report of this work has appeared [8].

Materials and Methods

Reduced dithiothreitol was purchased from Sigma and oxidized dithiothreitol was prepared according to the method of Cleland [9]. All other chemicals used were Merck (Darmstadt, Germany) reagent-grade products.

Crystalline bovine rhodanese was prepared from kidney as previously reported [10] and from liver as described by Horowitz and De Toma [11]. As crystallization had taken place in the presence of 1 mM thiosulfate the crystalline rhodanese was in the sulfur-containing form, i.e. the persulfide enzyme. Most of the experiments have been done on the beef kidney enzyme and comparative studies with the beef liver enzyme have shown no differences. Enzyme activity was measured by determining thiocyanate formation from thiosulfate and cyanide [12]. One enzyme unit is defined as the amount of protein converting 1 μmol of cyanide to thiocyanate per minute (pH 8.65, 20° C). Sulfur-free rhodanese was prepared as reported earlier [13]. Protein concentration was determined from the absorbance at 280 nm using a molar absorptivity $\epsilon = 64.75 \cdot 10^3$ and a molecular weight of 37000 [14].

Crystalline enzyme (600 units/mg) was dissolved in 50 mM Tris/acetate buffer, pH 8, and extensively dialyzed against the same buffer.

The reaction of dithiothreitol with rhodanese both in the sulfur-containing and in the sulfur-free forms was followed, in a stoppered cell of 1 cm pathlength, recording the increase of absorbance at 310 nm upon the addition of reduced dithiothreitol with a Beckman Acta III spectrophotometer equipped with a temperature control unit. Spectral curves in the 300—400 nm region were registered before and 20 min after the addition of reduced dithiothreitol to the enzyme. The molar ratio between enzyme and dithiothreitol was 1 : 2.

The experimental procedure for disulfide reduction with dithiothreitol was as reported by Iyer and Klee [15]. A few μl of 20% SDS, at pH 8, were added to the reaction mixture in order to unfold the protein. During these experiments the temperature was kept at 10° C and fresh reduced dithiothreitol solution was prepared immediately before use.

The assays of rhodanese-catalyzed thiosulfate reduction by dithiothreitol were carried out in 1 cm light-path quartz cuvettes containing 150 μmol Tris/acetate buffer, pH 8, 30 μmol thiosulfate and 60 μmol reduced dithiothreitol in 3 ml final volume. The reaction was started by adding 0.2 mg of rhodanese. The reaction rate was measured by recording the increase at 285 nm due to the appearance of oxidized dithiothreitol as a function of time. The amount of oxidized dithiothreitol formed was calculated using $\epsilon = 275\ M^{-1} \cdot cm^{-1}$ [9]. The autoxidation of dithiothreitol during the assay was negligible since before the enzyme addition the recorder traces showed a zero slope.

Polarographic analyses were performed with a 448 AMEL oscillopolarograph using a dropping mercury electrode. A 10-ml polarographic cell equipped for deaeration by nitrogen flow was used. The range of applied potential on the

dropping mercury electrode was from −0.2 to −1.2 V. The use of this technique allows a very accurate measure of concentration of dithiothreitol in the reduced or in the oxidized form and of HS^- since they give well-defined peaks in the range of the applied potential. In fact, in the above-mentioned experimental conditions, they show a potential peaks at −0.31, −0.93 and −0.29 V respectively. All these potentials are relative to the satured calomel electrode.

Standard curves have been drawn for each compound with an accuracy of 2%. The reaction was followed at room temperature, recording the disappearance of reduced dithiothreitol and the simultaneous appearance of oxidized dithiothreitol and HS^- after addition of stoichiometric amounts of rhodanese. The enzymic reduction of thiosulfate was assayed at room temperature using 0.2 mM of both thiosulfate and dithiothreitol. The reaction was started by adding 0.2 mg of rhodanese. The variation of electroreducible species was checked every 10 min throughout the course of the reaction.

The inactivation experiments were performed at 20° C incubating rhodanese and dithiothreitol at different molar ratios (1/1, 1/10 and 1/100) in 50 mM Tris/acetate buffer, pH 8.6. Enzyme concentrations were from 1 μM to 100 μM. Aliquots were taken from the reaction mixtures at appropriate time intervals and activity was determined in the standard assay [12]. The inactivation was expressed as percentage of residual activity relative to the untreated enzyme.

Results

Reaction of rhodanese with dithiothreitol

When rhodanese, at concentrations between 0.4 and 0.8 mM, was treated with dithiothreitol the absorption at 335 nm, due to the presence of a persulfide group, is completely removed with concomitant formation of oxidized dithiothreitol and sulfide. The absorption spectra of dithiothreitol-treated enzyme and that of sulfur-containing enzyme are reported in Fig. 1. The reduction of the persulfide group by dithiothreitol can also be followed either by recording the increase in absorbance at 310 nm due to the formation of oxidized dithiothreitol or by detecting the appearance of oxidized dithiothreitol and HS^- by the polarographic technique. However due to the spectral overlap between the increasing absorption at 310 nm and the decreasing band at 335 nm a quantitation of this reaction is hardly possible. Nevertheless it is possible to remove the oxidized dithiothreitol formed by reduction with cyanide. In this case (Fig. 1,c) a further decrease in absorbance at 335 nm is obtained. The resulting spectrum is quite similar to that of the sulfur-free enzyme [10]. On the other hand quantitative determination of oxidized dithiothreitol can be obtained by polarographic experiments. The addition of enzyme to a stoichiometric amount of reduced dithiothreitol causes the appearance of equivalent amounts of HS^- at −0.29 V and oxidized dithiothreitol at −0.93 V (Fig. 2a, b). In this way it was possible to titrate the amount of sulfur bound to the enzyme by determining polarographically the increase of oxidized dithiothreitol and HS^- peaks after each reduced dithiothreitol addition. The titration stops when no further increase of the reaction products is observed and the reduced dithiothreitol peak becomes apparent. In these conditions (Fig. 2,c) the

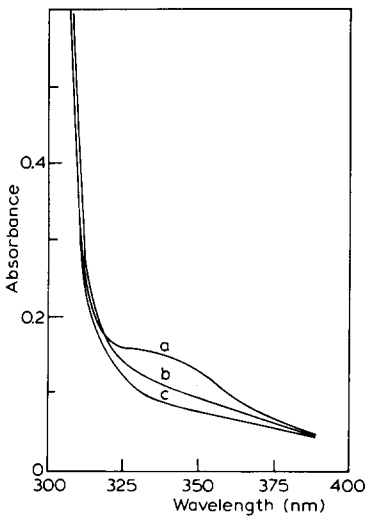

Fig. 1. Absorption spectra of rhodanese. The experiments was carried out in 50 mM Tris/acetate, 1 mM EDTA, pH 8, at 10°C. 0.5 mM rhodanese (sulfur-containing form) as such: curve a; in the presence of 1 mM reduced dithiothreitol: curve b and after addition of 10 mM cyanide to the dithiothreitol-treated enzyme: curve c.

reduction of the rhodanese persulfide group by dithiothreitol gives sulfide and oxidized dithiothreitol in amounts corresponding to 1.4 atoms of sulfur per mol of enzyme. These results fit with the following reaction:

$$E\text{—}S\text{—}SH + DTT_{red} \rightarrow E\text{—}SH + HS^- + DTT_{ox}$$

taking into account that by other titration methods [16] it is also possible to titrate only 1.35 atoms of persulfide sulfur per mol of enzyme. After this treatment the enzyme has full catalytic activity. The sulfur-free rhodanese does not react with dithiothreitol. However if thiosulfate is added to this reaction mixture the protein persulfide group is regenerated and a 310 nm absorbance is obtained which accounts for oxidized dithiothreitol formation. After the persulfide reduction no further disulfide bonds in the enzyme could be reduced by dithiothreitol even with prolonged incubation with 0.2% SDS in order to unfold the enzyme. Similar results were obtained with sulfur-free enzyme.

Thiosulfate reduction catalyzed by rhodanese in the presence of dithiothreitol

The addition of 20 mM reduced dithiothreitol to the incubation mixture containing 10 mM thiosulfate and 2 μM rhodanese in 50 mM Tris/acetate buffer, pH 8, causes the reduction of thiosulfate according to the reaction:

$$DTT_{red} + S_2O_3^{2-} \rightarrow DTT_{ox} + SO_3^{2-} + HS^-$$

The enzymic activity was followed spectrophotometrically by measuring the rate of dithiothreitol oxidation at 285 nm. Under these conditions 0.15 μmol/min of oxidized dithiothreitol were formed. In the absence of rhodanese no reaction occurred between dithiothreitol and thiosulfate. Thiosulfate reductase

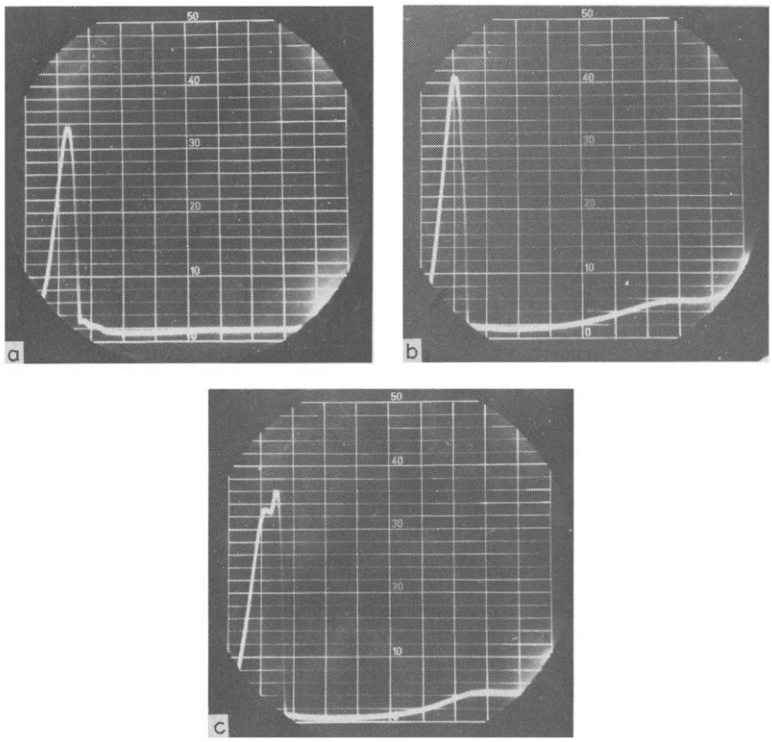

Fig. 2. Oscillopolarograms of dithiothreitol reaction with rhodanese. The supporting electrolyte was 50 mM Tris/acetate buffer, pH 8. Abscissa: applied potential from —0.2 to —1.2 V via saturated calomel electrode. Ordinate: current due to electroreducible species (μA). (a) peak at —0.31 V due to 60 μM reduced dithiothreitol and (b) its disappearance after addition of 60 μM rhodanese with appearance of two peaks corresponding to equivalent amounts of HS^- at —0.29 V and oxidized dithiothreitol at —0.93 V; current range 0.15 μA/div. (c) Complete reaction of 60 μM rhodanese after addition of 100 μM reduced dithiothreitol; current range 0.2 μA/div.

activity of rhodanese also carried out in the polarographic cell. At 10-min intervals the disappearance of reduced dithiothreitol and the appearance of HS^- and oxidized dithiothreitol were registered. However since HS^- peak at —0.29 V is too close to that of reduced dithiothreitol at —0.31 V to be resolved, an accurate quantitative determination of these two compounds during the enzyme reaction was not possible. For studying the reverse reaction cyanide was used instead of sulfite. Starting with equimolar amounts of HS^- and oxidized dithiothreitol (10 mM) in the presence of 25 mM cyanide, at pH 8, Tris/acetate buffer 50 mM, no reaction occurred in the absence of enzyme. When 2 μM rhodanese was added to the incubation mixture reduced dithiothreitol and SCN^- were formed according to the reaction:

$$DTTox + HS^- + CN^- \rightarrow DTTred + SCN^-$$

The enzymatic reaction was followed by testing thiocyanate formation with colorimetric analysis [12]. Under these conditions 0.05 μmol/min of thiocyanate were formed.

TABLE I

Residual activity of sulfur-containing rhodanese, as percentage of that of the untreated enzyme, after treatment with dithiothreitol in various molar ratios for 50 min. For experimental conditions see Methods.

[E]/[DTT_{red}]	[E]		
	100 μM	10 μM	1 μM
1 : 1	100	80	15
1 : 10	80	10	0
1 : 100	0	0	0

Rhodanese inactivation by dithiothreitol

The effect dithiothreitol on the sulfur transfer reaction catalyzed by rhodanese was investigated by incubating both sulfur-containing and sulfur-free enzymes in the presence of reduced dithiothreitol. In a typical experiment few μl of a reduced dithiothreitol solution, at pH 8, were added to 0.25 ml of 100 μM rhodanese in order to achieve molar ratios between enzyme and reduced dithiothreitol of 1 : 1, 1 : 10 and 1 : 100. As reported in Fig. 3 rhodanese is unaffected by dithiothreitol at a molar ratio 1 : 1 whereas at higher concentrations dithiothreitol produces a time-dependent decrease in enzyme activity which is a function of dithiothreitol concentration. These conditions require a 100-molar excess of reduced dithiothreitol to inactivate the enzyme rapidly and completely. The results obtained using different concentrations of enzyme

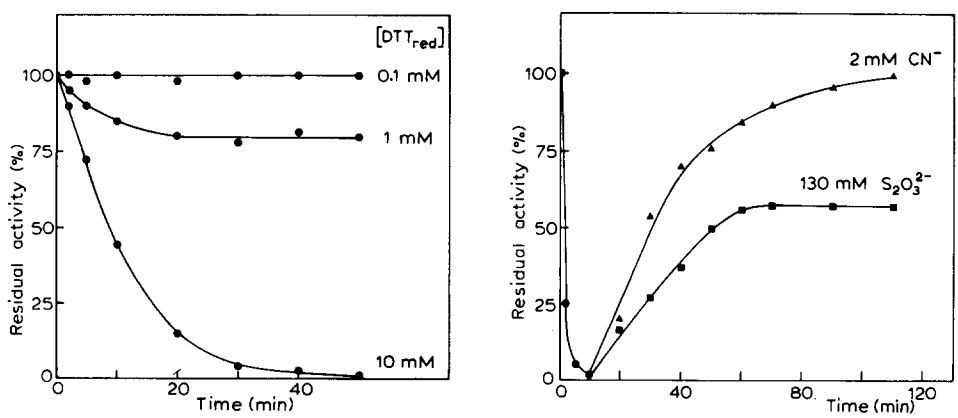

Fig. 3. Inactivation of rhodanese by dithiothreitol. 0.1 mM rhodanese (sulfur-containing form) was incubated at 20° C with different reduced dithiothreitol concentrations in 50 mM Tris/acetate buffer, pH 8.6. At the indicated times aliquots were taken from the incubation mixtures and tested for activity.

Fig. 4. Reversal of dithiothreitol inactivated rhodanese by cyanide or thiosulfate. 10 μM enzyme was incubated with a 100-molar excess of reduced dithiothreitol in 50 mM Tris/acetate buffer pH 8.6 at 20°C (●———●). After 10 min, when a complete inactivation was achieved, samples were treated immediately with cyanide to a concentration of 2 mM (▲———▲) or with thiosulfate to a concentration of 130 mM (■———■). Aliquots from the incubation mixtures were taken at the time indicated and activities were determined.

and dithiothreitol are reported in Table I. It appears that rhodanese is more sensitive to dithiothreitol inactivation at low protein concentration. Inactivation was not reversed by dialysis or gel filtration of the protein. The comparison of the inactivation experiments performed on sulfur-free rhodanese, in the same conditions reported for the sulfur-containing enzyme, shows at any time higher extent of inactivation (about 40%).

Reversal of dithiothreitol inactivation

The addition of thiosulfate or cyanide to the incubation mixture of 10 μM rhodanese after complete inactivation with 100 molar excess of reduced dithiothreitol, restored the enzyme activity. The reversal of dithiothreitol-inactivated rhodanese was time dependent. Cyanide restored the activity completely whilst thiosulfate only up 60% (Fig. 4).

Discussion

The use of reduced dithiothreitol has given useful information in the study of sulfur transfer reaction catalyzed by rhodanese. The presence of a persulfide group on the enzyme-sulfur complex [4] has found further support. The bleaching of the 335 nm absorption on addition of dithiothreitol is very fast and concomitant with the formation of oxidized dithiothreitol and sulfide. A disulfide bridge would have been cleaved more slowly without formation of sulfide. After reduction of the persulfide group no further reaction of dithiothreitol with the enzyme has been observed even a long time after the addition of SDS. These results indicate that no disulfide bonds are present in rhodanese molecule. This is in contrast with the hypothesis recently advanced by Man and Bryant [6] suggesting a disulfide group participation to the sulfur transfer. On the other hand the reactivitiy of dithiothreitol with the persulfide group, in contrast to the report of Wang and Volini [5], shows that dithiothreitol can be an active acceptor of sulfur in the reductive cleavage of thiosulfate by rhodanese. In fact the presence of thiosulfate in the incubation mixture containing reduced dithiothreitol and rhodanese, by restoring the persulfide group on the enzyme molecule, leads to continuous formation of oxidized dithiothreitol and sulfide. No inactivation of rhodanese was observed in the presence of thiosulfate nor when high concentrations of enzyme were used at enzyme/dithiothreitol ratio near 1. A rapid and complete inactivation by dithiothreitol, as reported by several authors [5,6,17] was instead observed in the absence of thiosulfate and with a 100-molar excess of dithiothreitol or when the enzyme was about 1 μM. Since no disulfide bonds are present in the rhodanese molecule and a free thiol group is essential for enzymic activity this inactivation could be due to the formation of a mixed disulfide between dithiothreitol and enzyme [17]. The involvement of the essential cysteinyl residue is supported by the protective effect of thiosulfate. The formation of a mixed disulfide between rhodanese and dithiothreitol as the inactivating event is confirmed by its reversal by addition of cyanide or thiosulfate. Both these compounds could reduce the dithiothreitol-enzyme disulfide. Our hypothesis on the mechanism of dithiothreitol inactivation is in agreement with a very recent report from Kim and Horowitz [17].

Acknowledgments

The authors are deeply indebted to Professor D. Cavallini for stimulating discussion and helpful criticism. We are also grateful to Mr. M. Achilli for technical assistance.

This work is part of a program supported by a grant from the Consiglio Nazionale delle Ricerche.

References

1 Green, J.R. and Westley, J. (1961) J. Biol. Chem. 236, 3047—3050
2 Westley, J. and Nakamoto, T. (1962) J. Biol. Chem. 237, 547—549
3 Westley, J. (1973) Adv. Enzymol. 39, 327—368
4 Finazzi Agrò, A., Federici, G., Giovagnoli, C., Cannella, C. and Cavallini, D. (1972) Eur. J. Biochem. 28, 83—93
5 Wang, S.F. and Volini, M. (1968) J. Biol. Chem. 243, 5465—5470
6 Man, M. and Bryant, R.G. (1974) J. Biol. Chem. 249, 1109—1112
7 Villarejo, M. and Westley, J. (1963) J. Biol. Chem. 238, 4016—4020
8 Cannella, C., Pecci, L., Pensa, B. and Costa, M. (1975) 10th FEBS Meeting 847
9 Cleland, W.W. (1964) Biochemistry 3, 480—482
10 Cannella, C., Pecci, L., Finazzi Agrò, A., Federici, G., Pensa, B. and Cavallini, D. (1975) Eur. J. Biochem. 55, 285—289
11 Horowitz, P. and De Toma, F. (1970) J. Biol. Chem. 245, 984—985
12 Sòrbo, B.H. (1953) Acta Chem. Scand. 7, 1129—1136
13 Cannella, C., Pecci, L., Costa, M., Pensa, B. and Cavallini, D. (1975) Eur. J. Biochem. 56, 283—287
14 Cannella, C., Pecci, L. and Federici, G. (1972) Ital. J. Biochem. 21, 1—7
15 Iyer, K.S. and Klee, W.A. (1973) J. Biol. Chem. 248, 707—710
16 Cannella, C., Pecci, L., Pensa, B., Costa, M. and Cavallini, D. (1974) FEBS Lett. 49, 22—24
17 Kim, S.K. and Horowitz, P.M. (1975) Biochem. Biophys. Res. Commun. 67, 433—439