HYDROGEN PEROXIDE INVOLVEMENT IN THE RHODANESE INACTIVATION BY DITHIOTHREITOL

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SUMMARY: The conditions required to obtain rhodanese inactivation in the presence of dithiothreitol indicate the involvement of hydrogen peroxide produced by metal-ion catalyzed oxidation of dithiothreitol. Inhibition of dithiothreitol oxidation by a chelating agent, or by removal of hydrogen peroxide by catalase prevents the enzyme inactivation. The inactivated enzyme contains a disulfide bond resulting from the oxidation of the catalytic sulfhydryl group and another sulfhydryl group close to it. This disulfide might be formed via a sulfenic intermediate.

Recent studies indicate that rhodanese (thiosulfate:cyanide sulfurtransferase EC 2.8.1.1.) is inactivated by DTT (1,2). Since the enzyme contains only reduced sulfhydryl groups and the formation of a disulfide bond is not required in the catalytic mechanism it seems unlikely that an efficient reducing agent would directly cause inactivation. However rhodanese as purified is an intermediate enzyme-sulfur complex (3) with substrate sulfur bound to a cysteinyl residue at the active site as a persulfide group (4). As we have recently shown (2) DTT reduces this persulfide group with formation of sulfide and oxidized DTT. The resulting sulfur--free enzyme retains its activity if a sulfur donor which restores the persulfide group is present; otherwise the enzyme is rapidly inactivated by excess DTT. The protective effect exerted by substrates (2) strongly suggests chemical modification of the essential sulfhydryl group upon DTT inactivation. On the other hand

Abbreviations: DTT - dithiothreitol; SDS - sodium dodecyl sulfate; EDTA - ethylenediamine tetraacetic acid.

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DTT at low concentration undergoes a metal-ion catalyzed oxidation with formation of superoxide ion which can dismute producing oxygen and hydrogen peroxide (5). This compound could oxidize one or more critical residue at the enzyme active site (6). The purpose of this work was to investigate whether the enzyme inactivation is due to DTT directly reacting with the enzyme, as suggested by Kim and Horowitz (1), or to compounds (like hydrogen peroxide, superoxide ion or other radicals) generated during the autoxidation of DTT. To discriminate between these hypotheses the effect of other hydrogen peroxide producing systems on rhodanese has been studied. A preliminary report of this study was already presented (7).

MATERIALS AND METHODS: Chemicals were reagent grade mostly from Merck. DTT in the reduced form was purchased from Sigma while its oxidized form was prepared according to Cleland (8). Beef liver catalase and horse-radish peroxidase were from Boehringer. Bovine erythrocyte superoxide dismutase was a kind gift from Dr L. Calabrese. Rhodanese was purified from beef liver as described by Horowitz and De Toma (9). Crystalline rhodanese was dissolved in 50 mM Tris-acetate buffer, pH 8.5 and then dyalized overnight against the same buffer. The enzyme (20 μM) was incubated at 20° with different concentrations of DTT in a final volume of 0.2 ml. The molar ratio between enzyme and DTT was from 1:100 to 1:10000. The same experimental procedure was followed when the enzyme was directly incubated with hydrogen peroxide, in a molar ratio varing from 1:1 to 1:25, or with 7 mM ascorbate. To eliminate excess DTT from the inactivated enzyme the incubation mixture was sieved on a column (1x30 cm) of Sephadex G 50 fine equilibrated with 50 mM Tris-acetate buffer, pH 8.5. Fractions containing protein were collected and then treated with 3000 fold excess of sodium borohydride. After complete destruction of borohydride the reaction mixture was analyzed with a 448 AMEL oscillopolarograph using a dropping mercury electrode. Other experimental procedures were as reported in a previous paper (2). The sulfhydryl content of rhodanese before and after inactivation was determined by titration with 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of 8 M urea essentially as described by Ellman (10). The procedure for SDS-polyacrylamide gel electrophoresis was as reported by Weber and Osborn (11) but using SDS in the sample solution only (12). In these conditions rhodanese shows a single band corresponding to a molecular weight of 35000.

RESULTS AND DISCUSSION: As previously reported, the treatment of rhodanese with DTT at high DTT/enzyme ratios or at very low enzyme

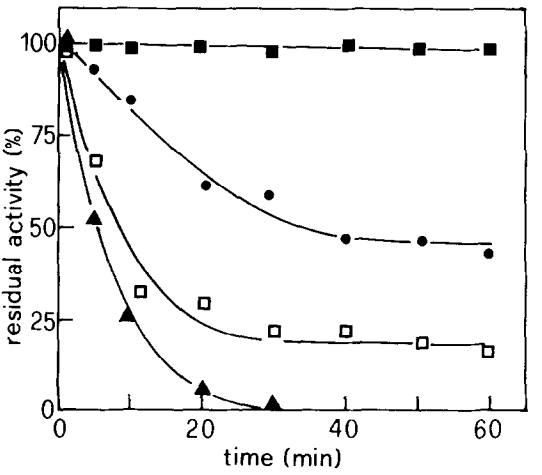
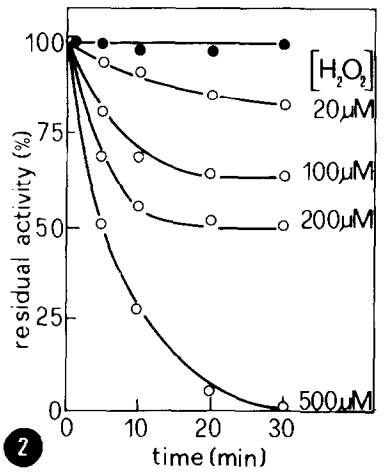


Figure 1: Effect of DTT on rhodanese activity.
20 μM rhodanese was incubated with different DTT concentrations in 50 mM Tris-acetate buffer pH 8.5 at 20°C (final volume 0.2 ml). At the times indicated 5 μl aliquots were withdrawn and assayed for activity according to Sorbo (23).
DTT concentrations: ▲2 mM, □20 mM, ●100 mM, ■200 mM.

concentration, leads to a complete inactivation (2). In the present work, using even higher DTT/enzyme ratios (i.e. higher than 100), less inactivation than expected is observed. Figure 1 shows that after 30 min incubation of rhodanese with 2 mM DTT a complete inactivation is obtained whereas at higher DTT concentration only a partial inactivation is achieved. When the concentration of DTT is further increased to 200 mM the enzyme retains its full activity. Also when the incubation mixtures are deaerated by flushing nitrogen no inactivation is observed in any experimental conditions. No inactivation is obtained with oxidized DTT. Thus rhodanese inactivation depends on the simultaneous presence of both oxygen and DTT in appropriate concentration. This result is particularly interesting in view of the reported metal-ion catalyzed oxidation of DTT with production of superoxide ion and hydrogen peroxide (5,6). These products might inactivate rhodanese. Consistent with this concept is the finding that high concentra-



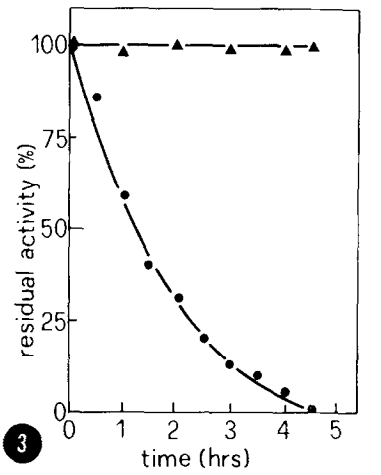
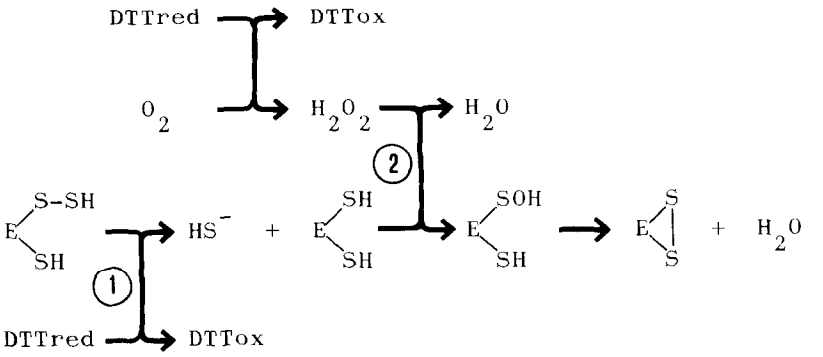


Figure 2: Effect of hydrogen peroxide on rhodanese activity. Experimental conditions as reported in Figure 1. Open circles: different hydrogen peroxide concentrations; solid circles: 500 μM hydrogen peroxide in the presence of 200 mM DTT.

Figure 3: Effect of ascorbate on rhodanese activity. In a final volume of 0.2 ml 14 μM rhodanese was incubated with 7 mM ascorbate and 20 μM $CuCl_2$ in 50 mM Tris-acetate buffer pH 8.5 at 20° in presence (▲) or in absence (●) of 0.8 μM catalase. At the times indicated 5 μl aliquots were taken from the incubation mixture and tested for activity.

tions of DTT are unable to inactivate rhodanese, since in these conditions DTT can chelate metal ions essential for the oxidation. This hypothesis is also supported by the observation that 90 mM EDTA protects rhodanese against DTT inactivation. It is important to note that EDTA has no protective effect when hydrogen peroxide is present nor shows any influence on the rhodanese activity. This appears to indicate that metal chelation prevents formation of hydrogen peroxide in DTT solutions rather than protecting the enzyme against hydrogen peroxide. The involvement of hydrogen peroxide in the mechanism of enzyme inactivation by DTT is supported by the ability of low concentrations of catalase (0.8 μM) to prevent it. Peroxidase too, in the presence of an ap-

propriate hydrogen donor, exerts a similar protection. The failure of superoxide dismutase to protect the enzyme indicates that hydrogen peroxide rather than superoxide ion is the oxidizing agent responsible for rhodanese inactivation. Similarly the hydroxyl radical scavenger (13) formate (2 mM) does not show any effect. Further evidence is obtained by treating enzyme with hydrogen peroxide or with other hydrogen peroxide producing systems. The incubations of rhodanese with different concentrations of hydrogen peroxide shows (Figure 2) that a 25 molar excess of hydrogen peroxide produces a time-dependent inactivation identical to that produced by 100 molar excess of DTT. It is interesting to note that the effect of hydrogen peroxide on the enzyme is completely abolished by addition of a large excess of DTT. This result suggests that another cause of the failure of high concentration of DTT to inactivate rhodanese might be the reaction of the hydrogen peroxide with excess of DTT rather than with a residue of protein. Further support for the hypothesis of hydrogen peroxide involvement comes from experiments made in the presence of ascorbate. As reported by Cavallini (14) and recently by Halliwell and Foyer (15) ascorbate is oxidized in the presence of Cu^{++} with formation of hydrogen peroxide which in turn may oxidize other substrates like pyruvate. Figure 3 shows that the incubation of rhodanese with 7 mM ascorbate and 20 μM $CuCl_2$ leads to a time-dependent inactivation of enzyme. If catalase is present in the incubation mixture no inactivation is apparent as shown in the same figure. Kim and Horowitz proposed that the binding of DTT to the enzyme is responsible for rhodanese inactivation (1). This possibility is ruled out by the following evidence. A DTT inactivated sample of enzyme (20 μM) was freed from excess reagent by gel filtration. The eluted protein was treated with sodium borohydride and then analyzed polarographycally. No peak at -0.31 V was observed indicating no release of free DTT from the enzyme. In these experimental conditions 1 μM reduced DTT was easily determined. Experiments were also car-



Scheme I

ried out to measure the number of sulfhydryl groups of rhodanese before and after inactivation by DTT. The decrease in number of sulfhydryl groups from 4 to 2 after DTT treatment indicates that inactivation may result from formation of a disulfide bond in the enzyme molecule. This result fits well with the previously reported findings (2) that inactivated enzyme can be reactivated by treatment with cyanide or thiosulfate. SDS-polyacrylamide gel electrophoresis excludes the formation of intermolecular disulfide bridges since no higher molecular weight forms are detected. On the other hand many authors (16-18) suggest the formation of intramolecular disulfide bridges after treatment of rhodanese with different reagents. The proximity of another non essential sulfhydryl group to the catalytic one has been demonstrated in a recent study from this laboratory (19). Some authors suggested that the first step in disulfide formation is the oxidation of a sulfhydryl group to sulfenic acid (20-22). To check this possibility we treated inactivated enzyme with 4000 molar excess of sodium arsenite. It has been proposed that this very mildly reducing compound may reduce sulfenic acids, but not disulfides, to thiols (20). The addition of arsenite to inactivated rhodanese, after gel filtration to remove excess DTT, causes a recovery of about 40% of activity in 180 min. Furthermore this reactiva-

tion occures only if rhodanese is not 100% inactivated. This result can be interpreted by supposing that a first step in the oxidation of enzyme is the formation of a sulfenic group, and that this subsequently reacts with a neighbouring sulfhydryl group to give a disulfide bridge no more susceptible to arsenite reduction (see scheme I). Thus it may be concluded, in agreement with our previous work (2), that the reaction of rhodanese with DTT consists in reduction of its persulfide group (reaction 1) while the enzyme inactivation is not due to DTT itself but to the hydrogen peroxide generated during its autoxidation (reaction 2). This oxidizing agent inactivates rhodanese by the formation of a disulfide bond between the catalytic sulfhydryl group and another one close to it in the active site possibly through the intermediate formation of a sulfenic group.

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REFERENCES

1. Kim, S.K. and Horowitz, P.M. (1975) Biochem.Biophys.Res.Commun. 67,433-439.
2. Pecci, L., Pensa, B., Costa, M., Cignini, P.L. and Cannella, C. (1976) Biochim.Biophys.Acta 445,104-111.
3. Westley, J. and Nakamoto, T. (1962) J.Biol.Chem. 237,547-549.
4. Finazzi Agrò, A., Federici, G., Giovagnoli, C., Cannella, C. and Cavallini, D. (1972) Eur.J.Biochem. 28,83-93.
5. Trotta, P.P., Pinkus, L.M. and Meister, A. (1974) J.Biol.Chem. 249,1915-1921.
6. Misra, H.P. (1974) J.Biol.Chem. 249,2151-2155.
7. Cannella, C., Pensa, B., Costa, M. and Pecci, L. (1976) Abstract II° Congr.Soc.It.Biochim. p. 141.
8. Cleland, W.W. (1964) Biochemistry 3,480-482.
9. Horowitz, P. and De Toma, F. (1970) J.Biol.Chem. 245,984-985.
10. Ellman, G.L. (1959) Arch.Biochem.Biophys. 82,70-77.
11. Weber, K. and Osborn, M. (1969) J.Biol.Chem. 244,4406-4412.
12. Stoklosa, J.T. and Latz, H.W. (1975) Anal.Biochem. 68,358-370.
13. Neta, P. and Dorfman, L.M. (1968) Advan.Chem.Ser. 81,222-230.
14. Cavallini, D. (1946) Arch.Fisiol. XLV,189-202.
15. Halliwell, B. and Foyer, C.H. (1976) Biochem.J. 155,697-700.
16. Baillie, R.D. and Horowitz, P.M. (1976) Biochim.Biophys.Acta 429,383-390.
17. Guido, K. and Horowitz, P.M. (1976) Biophys.J. 16,203a.

18. Weng, L., Heinrikson, R.L. and Westley, J. (1976) Fed.Proc. 35,1630.
19. Pensa, B., Costa, M., Pecci, L., Cannella, C. and Cavallini, D. (1977) Biochim.Biophys.Acta (in press).
20. Parker, D.J. and Allison, W.S. (1969) J.Biol.Chem. 244,180-189.
21. Little, C. and O'Brien, P.J. (1969) Eur.J.Biochem. 10,533-538.
22. Lin, W.S., Armstrong, D.A. and Gaucher, G.M. (1975) Can.J.Biochem. 53,298-307.
23. Sorbo, B.H. (1953) Acta Chem.Scand. 7,1129-1136.