A Study of Dithiothreitol Inactivation of the Enzyme Rhodanese

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Summary

When air oxidized, partially inactivated rhodanese (EC 2.8.1.1) is treated with dithiothreitol (DTT) to regenerate the reduced essential sulfhydryl group there is an initial reactivation followed by an anomalous slower inactivation. Fully active enzyme shows only inactivation. The inactivated enzyme may be completely reactivated on long incubation with the substrate thiosulfate ion. None of the normal potentialities of DTT appear to be responsible for the inactivation. The results are interpreted in terms of disulfide formation between DTT and an essential enzymic sulfhydryl group with the resulting complex being stabilized by secondary interactions which are particularly favorable due to similarities between DTT and lipoic acid--a normal sulfur acceptor substrate.

Introduction

The enzyme rhodanese (EC 2.8.1.1) will catalyze the reduction of thiosulfate using reduced lipoic acid as a sulfur acceptor and produce sulfite, sulfide, and oxidized lipoic acid (1). Fully active enzyme contains only reduced sulfhydryl groups and it is often found that purified rhodanese loses activity on storage due at least partially to the oxidation of essential sulfhydryl groups to disulfides (2).

When reactivation of oxidized rhodanese is attempted with dithiothreitol (DTT)[1] which reduces disulfides and is innocuous with free sulfhydryl groups (3) it is found that the enzyme is rapidly inactivated (4,5).

DTT can affect enzyme systems in at least two ways distinct from disulfide cleavage: Autooxidation of DTT produces hydrogen peroxide which can oxidize critical residues (6) and DTT by virtue of its dithiol structure can chelate metal ions (7).

[1] abbreviations: DTT, dithiothreitol.

These results are especially interesting in view of the susceptibility of rhodanese to oxidative inactivation (2) and the reported importance of metal ions in the catalytic mechanism (8).

We report here studies of the effect of DTT on rhodanese activity to attempt to elucidate the mechanism for the observed inactivation.

Experimental Procedure and Results

Crystalline bovine liver rhodanese was prepared by the method of Horowitz and DeToma (9). DTT was obtained from Calbiochem. All other materials were the best available commercial grades and were used as obtained.

Enzyme activity was measured by the method of Wang and Volini (10). Protein concentrations were measured using a modified biuret procedure (11).

For experiments requiring deaeration, nitrogen was bubbled through the buffers for 20 minutes. Microliter quantities of enzyme were then added and the solutions were blanketed with nitrogen throughout the experiment.

For the inactivation experiments crystalline rhodanese (0.74 IU/μg) was dissolved in 0.2 M Tris buffer pH 7.9[2] at 0° to give an enzyme concentration of 3×10^{-5} M (based on biuret protein and assuming a molecular weight of 32,600 for the enzyme). Microliter quantities of 0.1 M DTT were added at zero time to 1 ml of the prepared enzyme solution. Both the original enzyme solution which was used as the control and the enzyme solution with DTT were kept at 0°C and assayed as a function of time.

Figure 1 shows the time course of the activity of rhodanese samples with added DTT relative to controls without DTT. Curve 3 shows that when an air oxidized, partially inactivated enzyme sample (0.51 IU/μg) is treated with DTT at a molar ratio of 9:1, DTT:enzyme, there is a reactivation followed by a slower inactivation which is essentially complete by 70 minutes. Curve 4 shows that raising the DTT:enzyme ratio to 200:1 greatly increases the rate of inactivation. Curve 5 shows that fully active rhodanese (0.73 IU/μg)

[2]This buffer was used for all the studies presented here.

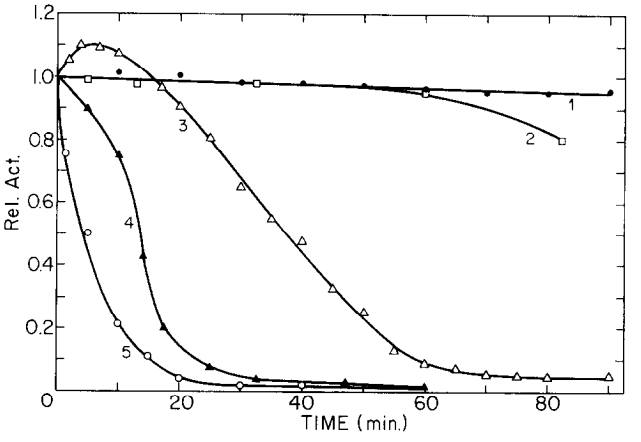


Figure 1. Time course of rhodanese inactivation with dithiothreitol. Curve 1, DTT:rhodanese = 10:1, deaerated; Curve 2, β-mercaptoethanol:rhodanese = 9:1; Curve 3, DTT:rhodanese = 9:1, specific activity = 0.51 IU/μg; Curve 4, same as curve 3 with DTT:rhodanese = 200:1; Curve 5, DTT:rhodanese = 200:1, specific activity = 0.73 IU/μg. See text for experimental details.

treated with a 200 fold molar excess of DTT displays only rapid inactivation.

Curve 2 of Figure 1 shows as a point of comparison that the monothiol β-mercaptoethanol at a 9 fold molar excess over enzyme gives only a small degree of inactivation during an 80 minute incubation. This curve can be compared with curve 3 for the same molar excess of DTT. It may be noted in addition that when DTT was oxidized before addition to the enzyme sample, there was no effect on the enzyme activity at any dithiothreitol:enzyme ratio up to and including 100:1.

Curve 1 of Figure 1 shows the effect when the system was deaerated and a 10 fold molar excess of DTT over enzyme added. In this case no inactivation of the enzyme was apparent. This result appears to eliminate the possibility that inactivation results from the chelation of an essential metal ion and indicates that an oxidation is required.

Trotta, et al. have indicated that low concentrations of DTT can pro-

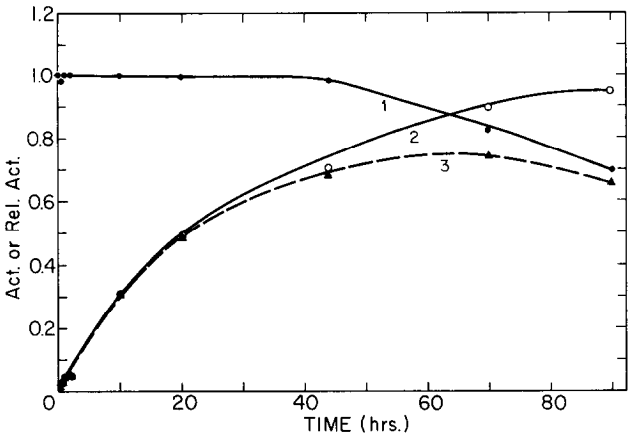


Figure 2. Time course of thiosulfate reactivation of DTT inactivated rhodanese. Rhodanese concentration = 0.91 mg/ml. Curve 1, activity of control; Curve 2, activity of reactivating sample relative to control; Curve 3, activity of reactivating sample. For curves 1 and 3 an ordinate value of 1 corresponds to an optical density of 0.5 in the standard assay. See text for experimental details.

duce hydrogen peroxide on autooxidation which can irreversibly inactivate susceptible enzymes. This effect could be abolished by using high DTT concentrations (6). In the present case incubation of rhodanese (0.85 mg/ml, 0.71 IU/μg) in 0.2 m$\underline{M}$ hydrogen peroxide gave less than 30% inactivation of the enzyme relative to a control without added hydrogen peroxide after two hours of incubation. Incubation of active enzyme (0.35 mg/ml, 0.71 IU/μg) with 20 m$\underline{M}$ DTT rather than stabilizing the enzyme resulted in complete loss of activity in less than ten minutes. These last results differ markedly from those of Trotta, et al. and eliminate hydrogen peroxide production as the complete explanation of the phenomena observed here.

Figure 2 shows that enzyme that has been inactivated by DTT can be completely reactivated, relative to a control, by long incubation at 4°C in the presence of 62.5 m$\underline{M}$ sodium thiosulfate. The activity of the reactivating enzyme relative to the control is shown by curve 2 and begins at

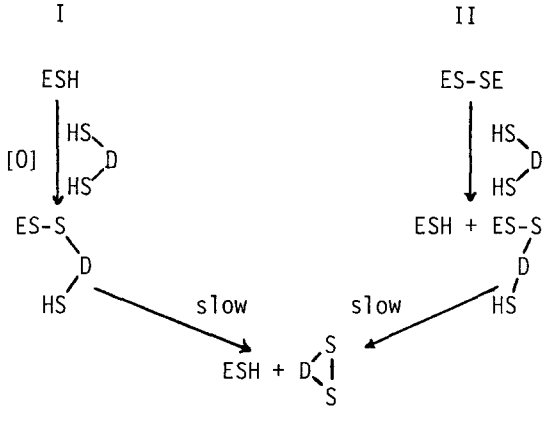
zero relative activity at t=0 and rises to 96% of the control values at 90 hours. Figure 2 also shows the individual time courses for the control (curve 1) and the reactivating sample (curve 3). An ordinate value of 1 for these two curves corresponds to an OD = 0.50 in the assay system.

In addition the rate of inactivation by DTT may be slowed by including the substrate thiosulfate in the incubation mixture. When the molar ratio of thiosulfate:DTT was 400:1 or greater, there was no inactivation during a 90 minute incubation (DTT:enzyme = 8:1).

Discussion

In the past it has often been assumed that when DTT affected enzyme activity this indicated the involvement of a disulfide bond. DTT is in fact a potent disulfide reductant but may also chelate metal ions and produce hydrogen peroxide. The results presented here indicate that none of these potentials is realized in the inactivation of rhodanese.

It appears that a direct interaction between DTT and the enzyme is responsible for the observed inactivation. Presented below is a mnemonic model for the effects we have described:



Scheme II shows rhodanese inactivated by the oxidation to a disulfide of two essential sulfhydryl groups from separate active sites (2). This inactive species may interact with a molecule of DTT to participate in a disulfide-sulfhydryl group interchange, releasing one active enzyme unit and producing

an enzyme-DTT complex. This represents a reactivation step. Normally DTT would rapidly cyclize in the last step to produce the stable oxidized ring form of DTT thus completing the reactivation. It is suggested that stabilization of the complex occurs, using the secondary interactions responsible for binding the substrate reduced lipoic acid--a 1,3 aliphatic dithiol that forms a kinetically significant complex with rhodanese (1). Therefore, the last step in this scheme is shown as limiting the rate at which the enzyme may be fully reactivated. It is interesting that in the absence of thiosulfate, lipoic acid will also inactivate the enzyme (12).

None of the steps above require any net oxidation and the necessity for oxygen is suggested in Scheme I. Active rhodanese with its free active site sulfhydryl group is shown at the top. Under oxidizing conditions there can be disulfide formation to give the DTT-enzyme complex directly, which again would be stabilized (cf. Scheme II). This cannot readily occur on deaeration. Only inactivation would be observed in this sequence in the presence of oxygen.

Acknowledgment

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