Mechanism of Rhodanese Action: Isotopic Tracer Studies*

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In a previous publication (2) a double displacement mechanism for the catalytic activity of rhodanese (thiosulfate cyanide sulfurtransferase) was proposed on the basis of polarographic evidence. In addition it was concluded from the polarographic data that crystalline beef liver rhodanese is an enzyme-substrate intermediate containing two activated substrate sulfur atoms per molecule. This communication reports the results of sulfur-35 tracer experiments which support the previous conclusions. The splitting of labeled thiosulfate and the specific binding of the transferable sulfur atom by rhodanese have been demonstrated. Studies on the removal of the substrate sulfur from the enzyme have provided some additional data concerning the nature of this enzyme-substrate intermediate.

EXPERIMENTAL PROCEDURE

Crystalline beef liver rhodanese was prepared as previously described (3). The rhodanese assay procedure of Sörbo (4) has been modified for convenience by reducing all volumes by 60%, elevating the temperature to 25°, and decreasing the reaction time to 1 minute. The rhodanese unit has been retained as originally defined (4). Radioactivity values were determined for samples of negligible self-absorption on aluminum or stainless steel planchets in a windowless gas flow counter with the use of helium saturated with ethanol at 0° as the gas mixture. Analytical zone electrophoresis was done using the system previously described (5). The supporting medium was rayon acetate satin fabric and the buffer was glycine-NaOH, pH 8.7, p = 0.04. The applied potential was 20 volts per cm. The duration of electrophoresis was 1 hour. After electrophoresis, the fabric was dried at 110° in a horizontal position and either stained with nigrosine to determine the protein distribution or cut into strips and placed on planchets for radioactivity determinations.

Thiosulfate labeled in the inner position (SSO₃⁻) was prepared from elemental sulfur-35 with Na₂SO₃ by the synthetic procedure described above. The isotopically labeled sulfite was precipitated from the reaction mixtures by the addition of ammonium sulfate to 2.4 M at pH 7.9, and washed twice as a precipitate with 3.25 M ammonium sulfate, pH 7.9. Analysis for S₅O₃⁻ content by the chemical method of Sörbo (7) gave an isotopic yield of 85%.

Enzymatic analysis, using the reaction catalyzed by rhodanese, yielded the same result. When this preparation was subjected to zone electrophoresis, at least 98% of the total radioactivity was accounted for in a single symmetrical band with the mobility of thiosulfate.

To verify the position of the radioactive isotope, an aliquot of the SSO₃⁻ solution was degraded to elemental sulfur and SO₂ by treatment with 3 M HCl. The precipitated amorphous sulfur was washed with water and allowed to dry and crystallize. In this form the sulfur dissolved readily in benzene. Aliquots of the benzene solution were transferred to planchets and evaporated to dryness for the determination of radioactivity. The elemental sulfur contained 1,063 c.p.m. or 99% of the radioactivity of the original SSO₃⁻ (1,685 c.p.m.). The SO₂ resulting from the acid decomposition of the SSO₃⁻ was swept out of solution with a stream of air and trapped in NaOH solution. This solution was acidified and BaCl₂ was added, followed by H₂O₂ and Fe³⁺ to oxidize the SO₂ to sulfate. Less than 1% (5 c.p.m.) of the radioactivity of the SSO₃⁻ was found in the precipitated BaSO₄.

Thiosulfate labeled in the inner position (SSO₃⁻) was prepared from elemental sulfur-32 and S₅O₃⁻ by the synthetic reaction described above. The isotopically labeled sulfite was prepared by combustion of elemental sulfur-35 in oxygen. The S₅O₃⁻ formed was trapped in dilute NaOH solution as SSO₃⁻. This solution was added directly to an excess of elemental sulfur-35 and the synthesis and analysis were carried out as before. The over-all isotopic yield of S₅O₃⁻ was 80%. Degradation of an aliquot of this preparation with acid, as described above, yielded the following results:

\[
\text{SSO}_{3}^{-} + H^+ \rightarrow \text{Elemental S} + \text{SO}_{3}^{2-}
\]

(1,707 c.p.m.) (20 c.p.m.)

\[
\text{H}_2\text{O}_2 \rightarrow \text{SO}_{3}^{2-}
\]

(1,710 c.p.m.)

RESULTS

Transfer of Thiosulfate Sulfur to Rhodanese—A series of experiments was done to detect exchange reactions occurring between thiosulfate and rhodanese. Crystalline rhodanese was dissolved in solutions containing isotopically labeled thiosulfate in 1 M glycine at 0°. A considerable range of specific radioactivities and ratios of substrate ion to enzyme was used. The enzyme was precipitated from the reaction mixtures by the addition of ammonium sulfate to 2.4 M at pH 7.9, and washed twice as a precipitate with 3.25 M ammonium sulfate, pH 7.9. The addition of a small volume of 1 M ammonium sulfate to the amorphous
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**TABLE I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate</th>
<th>Specific radioactivity of Rhodanese used</th>
<th>Rhodanese reisolated</th>
<th>Labeling of rhodanese with substrate sulfur-35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mc/mole</td>
<td>moles/mole</td>
<td>labeled sulfur/Rhodanese (corrected for 0.15% contamination)</td>
</tr>
<tr>
<td>A</td>
<td>SS4S03⁻</td>
<td>0.13</td>
<td>270</td>
<td>0.5</td>
</tr>
<tr>
<td>B</td>
<td>SS4S03⁻</td>
<td>0.25</td>
<td>110</td>
<td>0.3</td>
</tr>
<tr>
<td>C</td>
<td>SS4S03⁻</td>
<td>0.18</td>
<td>55</td>
<td>0.1</td>
</tr>
<tr>
<td>D</td>
<td>SS4S03⁻</td>
<td>8.7</td>
<td>200</td>
<td>1.6</td>
</tr>
<tr>
<td>E†</td>
<td>SS4S03⁻</td>
<td>0.23</td>
<td>185</td>
<td>1.9</td>
</tr>
<tr>
<td>F†</td>
<td>SS4S03⁻</td>
<td>0.44</td>
<td>110</td>
<td>1.7</td>
</tr>
<tr>
<td>G</td>
<td>SS4S03⁻</td>
<td>0.17</td>
<td>95</td>
<td>1.7</td>
</tr>
<tr>
<td>H</td>
<td>SS4S03⁻</td>
<td>0.22</td>
<td>60</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* The application of this correction probably gives ratios for the SS4S03⁻ experiments which are too low but they are included to show that such correction could not alter the qualitative conclusions. Contamination at a level of 0.15% is higher than expected on the basis of the isolation procedure and is probably occasioned by a quantitatively minor reaction between rhodanese and a trace contaminant in the SS4S03⁻ solution. A further recrystallization of a labeled rhodanese preparation from one SS4S03⁻ experiment reduced the contamination level by only 40%.† In these experiments, SO₃⁻ was also added to an extent of 0.5% of the SSO₃⁻ concentration.

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**TABLE II**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Inactivating agent</th>
<th>Conditions of treatment*</th>
<th>Recovery of S³⁻ (in %, recovered protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CN⁻</td>
<td>3°, 10 sec,† CN⁻/rhodanese = 100</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>CN⁻</td>
<td>3°, 1 min,† CN⁻/rhodanese = 10</td>
<td>7.5</td>
</tr>
<tr>
<td>C</td>
<td>SO₃⁻</td>
<td>3°, 1 min,† SO₃⁻/rhodanese = 100</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>Trichloroacetic acid</td>
<td>3°, 2 M acid</td>
<td>2</td>
</tr>
<tr>
<td>E</td>
<td>Heat</td>
<td>80°, 1 min</td>
<td>8.5</td>
</tr>
<tr>
<td>F</td>
<td>None</td>
<td>3°, 1 min</td>
<td>90</td>
</tr>
</tbody>
</table>

* Treatment of 50 μmoles of the twice recrystallized preparation H, Table I, in 0.10 ml of 0.5 M glycine-NaOH buffer, pH 8.6.
† Interval between addition of inactivating reagent and addition of (NH₄)₂S0₃ to 3 m to precipitate the protein.

**Fig. 1.** Electrophoretic distribution of sulfur-35 in rhodanese recrystallized after treatment with SS₄S0₃⁻. The point of application of the sample was between the final 3- and 4-cm positions. Zero centimeters represents the final position of the electro-osmotic marker. The major peak corresponds to the position of rhodanese. The absence of free SS₄S0₃⁻ is indicated by the absence of a maximum in the 8-cm region.

Precipitate caused the enzyme to crystallize (3, 4). The crystalline enzyme was recovered by centrifugation and dissolved in 0.1 M glycine. Assays for enzymatic activity were done as an index of protein content, on the basis of a specific activity of 200 rhodanese units per mg of protein for the recrystallized enzyme (3, 4). Recovery of enzyme in these experiments was 60 ± 10%. Aliquots of the solution of recrystallized rhodanese taken for radioactivity determinations (1 to 10 μl) were diluted, spread over an area of 19.6 cm², and dried for counting. From the known molecular weight of the enzyme (4) and the specific radioactivity values of the labeled thiosulfate used and the rhodanese reisolated, the number of labeled sulfur atoms transferred to each molecule of rhodanese was calculated.

The results of five such experiments utilizing SS₄S0₃⁻ and three utilizing SS₄O₃⁻ are given in Table I. From these results it is clear that there was a specific transfer of sulfur-35 to rhodanese from SS₄S0₃⁻ but not from SS₄O₃⁻. With the outer labeled substrate, approximately 2 atoms of labeled sulfur appeared for each molecule of reisolated enzyme, independent of the quantity of substrate added. With inner labeled substrate, however, there appeared only a contamination level of radioactivity, amounting to about 0.15% of the total sulfur-35 added. In this case, the quantity of labeled sulfur appearing in the reisolated enzyme bore no stoichiometric relationship to the quantity of the enzyme.

To verify the binding of labeled substrate sulfur to the enzyme,
rhodanese crystallized after treatment with $^{35}$SO$_4^{2-}$ was subjected to zone electrophoresis and the dried electrophoresis fabric was scanned for radioactivity. The distribution observed is shown in Fig. 1.

**Removal of Thiosulfate Sulfur from Rhodanese**—In a further series of experiments, crystalline rhodanese prepared as described above to contain sulfur-35 from $^{35}$SO$_4^{2-}$ was inactivated by treatment with CN$^-$, SO$_3^{2-}$ or trichloroacetic acid or by heating. The rhodanese was then recovered and assayed for radioactivity. The results of these experiments are presented in Table II. These data indicate that inactivation of the enzyme is accompanied by essentially complete loss of the activated substrate sulfur. In the first CN$^-$ experiment, the residual enzymatic activity in the recovered protein was also measured and found to be 13%, in good agreement with the recovery of sulfur-35 in the protein. In the second CN$^-$ experiment, carrier SCN$^-$ was added to the supernatant solution after precipitation of the protein. Silver thiocyanate was then precipitated by the addition of AgNO$_3$ in HNO$_3$. Despite difficulty in spreading this precipitate in a sufficiently thin layer on the planchet, more than 60% of the radioactivity originally present in the protein sample was detected in the AgSCN.

To confirm the identity of the products obtained when rhodanese reacts with CN$^-$ or SO$_3^{2-}$, crystalline enzyme containing sulfur-35 from $^{35}$SO$_4^{2-}$ was treated with these reagents and the whole reaction mixtures then subjected to zone electrophoresis. The completed electrophoresis strips were scanned for radioactivity. The results, shown in Fig. 2, were qualitatively unequivocal despite difficulty in mixing the microliter reaction volumes. Treatment with CN$^-$ produced $^{35}$CN$^-$ and treatment with SO$_3^{2-}$ produced $^{35}$SO$_4^{2-}$.

The supernatant solution containing substrate sulfur-35 released from the enzyme by heat denaturation was also subjected to zone electrophoresis but the radioactive material could not be eluted with 1 M HCl. The results presented support the double displacement mechanism of rhodanese action previously proposed (2):

$$\text{Rhodanese} + 2 \text{SSO}_4^{2-} \rightleftharpoons \text{rhodanese-S}_2 + 2 \text{SO}_3^{2-} \quad (1)$$
$$\text{Rhodanese-S}_2 + 2 \text{CN}^- \rightleftharpoons \text{rhodanese} + 2 \text{SCN}^- \quad (2)$$

These results are also a direct demonstration of the existence of crystalline rhodanese in the form rhodanese-S$_2$ of Equations 1 and 2, again in agreement with the polarographic evidence. When crystalline rhodanese is incubated with $^{35}$SSO$_4^{2-}$, a reaction occurs in which the enzyme becomes labeled with sulfur-35. In view of the polarographic evidence showing that rhodanese as isolated in crystalline form contains 2 activated substrate sulfur atoms per molecule (2), it is clear that this labeling reaction involves the equilibrium represented by Equation 1, in which crystalline rhodanese is the rhodanese-S$_2$ form. Although it would be predicted from Equation 1 that such an exchange could be abolished by complete exclusion of SO$_3^{2-}$ from the reaction mixture, this experiment cannot be done as thiosulfate solutions always contain a trace of free sulfite (6). In practice, the exchange reaction proceeds rapidly in the presence as well as in the absence of an additional trace of SO$_3^{2-}$ in the mixture of rhodanese-S$_2$ and $^{35}$SSO$_4^{2-}$ (Experiments D, E, F, and G, Table I). Isolated crystalline rhodanese-S$_2^{35}$ has been used to demonstrate Reaction 2 by showing the formation of $^{35}$SO$_4^{2-}$ from rhodanese-S$_2^{35}$ and SO$_3^{2-}$. These reactions occur very rapidly at ice-bath temperatures.

The value of the ratio (atoms of bound substrate sulfur)/ (molecule of rhodanese), in both the present work and the polarographic studies, is between 1.5 and 2. This presumably indicates the existence of two sites for transferable sulfur on each rhodanese molecule. The consistent finding of 5 to 15% less than the nearest integral value may indicate the presence of some rhodanese molecules containing single substrate sulfur atoms which can contribute to the enzyme assay values.

The demonstration of the removal of substrate sulfur from rhodanese when the enzyme is denatured adds to the conclusions reached from the polarographic evidence. The latter showed that the polarographic diffusion current given by the enzyme-bound substrate sulfur disappears on denaturation of the enzyme. The present work demonstrates that the sulfur is actually removed from the enzyme. Taken together, these facts constitute evidence regarding the chemical form of the released substrate sulfur. Free S$^-$, SSO$O_3^-$, SO$_3^{2-}$, thiol, and disulfide sulfurs all give polarographic currents, whereas the released substrate sulfur gives none. The chemical form of this sulfur could, therefore, be either colloidal elemental sulfur or some ion which neither forms complexes with the ions of Hg nor is reduced at the dropping Hg electrode. To distinguish between these alternatives, a solution containing labeled, released substrate sulfur was treated with a strong anion exchange agent and also subjected to zone electrophoresis. Both of these procedures resulted in irreversible precipitation of the radioactive material. As this behavior is that of colloidal sulfur rather than that of any ion, released substrate sulfur must be in the elemental form.

**SUMMARY**

Sulfur-35 tracer data have been obtained which strongly support the double displacement mechanism for rhodanese catalysis. Isolation of the crystalline enzyme-substrate intermediate containing 2 atoms of labeled sulfur per mole of enzyme from $^{35}$SSO$_4^{2-}$, but not from SS$^{34}$O$_4^{2-}$, has demonstrated directly that the intermediate is of the form rhodanese-S$_2$. An examination of the reactions of this labeled intermediate with CN$^-$ and SO$_3^{2-}$ has yielded data demonstrating the ready occurrence of the two reactions which constitute the postulated mechanism. Denaturation of rhodanese has been shown to release the activated substrate sulfur from the enzyme as colloidal elemental sulfur.

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**REFERENCES**
