Improved Assay for Rhodanese in *Thiobacillus* spp.

DAVID R. SINGLETON† AND DAVID W. SMITH*

School of Life and Health Sciences, University of Delaware, Newark, Delaware 19716

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Rhodanese (thiosulfate:cyanide sulfurtransferase; EC 2.8.1.1) catalyzes the conversion of thiosulfate and cyanide to thiocyanate and sulfate. Conventional rhodanese assays colorimetrically measure the formation of one or the other of the products. These assays suffer from the fact that there is significant nonbiological formation of these products in addition to the enzymatically catalyzed reaction. In the present report, we describe a modified procedure for assaying rhodanese in which a separate boiled control was prepared for each assay trial. The boiled control corrected for the nonbiological contributions to product formation.

Rhodanese (thiosulfate:cyanide sulfurtransferase; EC 2.8.1.1) catalyzes the transfer of the reduced sulfur atom in a sulfane-containing compound to a thiophilic acceptor. This enzyme is commonly assayed by using thiosulfate as the sulfur donor and cyanide as the acceptor, yielding sulfite and thiocyanate: \( \text{S}_2\text{O}_3^{2-} + \text{CN}^- \rightarrow \text{SO}_3^{2-} + \text{SCN}^- \). Subsequent reaction of thiocyanate with ferric ion produces ferric thiocyanate, which can be measured colorimetrically (3, 15). Alternatively, the other product of the reaction, sulfate, can be measured colorimetrically (14).

Rhodanese is widespread in the biological world (18), although its physiological role has been debated for many years, with proposals ranging from the detoxification of cyanide (3, 8) to suggestions that rhodanese is important in the bioenergetic oxidation of thiosulfate (16), to proposals that rhodanese may be significant in the pathways involved in generating iron-sulfur protein complexes and in lipopeptide metabolism (2, 4, 7, 17), to the recent demonstration that rhodanese catalyzes reactivation of nitrogenase (11).

In the standard rhodanese assay reaction, there are three thiocyanate-producing reactions which can occur: (i) the enzymatically catalyzed reaction between thiosulfate and cyanide; (ii) the nonenzymatic reaction between the same two substrates (5); and (iii) the spontaneous cyanoysis of elemental sulfur (1).

\[
\begin{align*}
\text{S}_2\text{O}_3^{2-} + \text{CN}^- & \xrightarrow{\text{rhodanese}} \text{SCN}^- + \text{SO}_3^{2-} \\
\text{S}_2\text{O}_3^{2-} + \text{CN}^- & \xrightarrow{\text{spontaneous}} \text{SCN}^- + \text{SO}_3^{2-} \\
\text{S}_2 + \text{CN}^- & \xrightarrow{\text{spontaneous}} \text{SCN}^{-}
\end{align*}
\]

Previous investigations have implicitly assumed that the first of these reactions was the only significant reaction occurring in the assay system, although Westley (18) cautioned against potential nonbiological interferences. Because most investigations into the physiological role of rhodanese in bacteria use the thiocyanate and related assays (3, 14), conclusions based on these assays must be considered questionable because of the contributions of the above-mentioned nonbiological reactions.

During the course of investigations of rhodanese activity in a marine strain of the bacterium *Thiobacillus intermedius*, it became apparent that there were difficulties with the standard assay procedure. In particular, it was determined that all three of the above reactions were occurring when rhodanese was assayed from *T. intermedius*. Therefore, a method was devised to determine the contributions of each of these reactions to total thiocyanate production.

The marine *T. intermedius* was originally isolated from a salt marsh in Lewes, Del., and was maintained in chemostat culture (BioFlo; New Brunswick Scientific Co.) fed with NaCl-amended thiosulfate mineral salts medium (D. Greenley, Ph.D. thesis, University of Delaware, Newark, 1982).

The initial assay procedure was an adaptation of that of Bowen et al. (3) which used untreated whole cells. Cells were removed aseptically from the chemostat and centrifuged at 15,000 × g for 15 min at 5°C. The pellet was suspended in assay buffer (0.067 M K2HPO4 plus 20 ppt of NaCl [20 g of NaCl per liter], pH 8.1) in a volume equal, in most cases, to 10% of the original sample removed from the vessel. A 0.5-ml portion of the cell suspension was added to 1.0 ml of assay buffer and 0.5 ml of thiosulfate (0.1 M Na2S2O3 plus 20 ppt of NaCl). Following a 10-min preincubation at 30°C, the reaction was begun by the addition of 0.5 ml of prewarmed cyanide solution (0.1 M KCN plus 20 ppt of NaCl). The reaction was stopped after desired time intervals by the addition of 0.25 ml of formaldehyde (HCHO) (37%, wt/vol). Color development was achieved by the addition of 2.75 ml of ferric nitrate solution [15%, wt/vol, Fe(NO3)3 in 1 N HNO3] to the reaction mixture, which was filtered through 0.22-μm membrane filters (Gelman Sciences, Inc.) to remove cellular debris. The optical density was measured at 470 nm. The spectrophotometer blank in these initial assays consisted of the complete assay system to which formaldehyde had been added before the cyanide. Several methods were tried to render the bacterial cells more permeable to the assay substrates and therefore increase the reaction rate: cold shock (12), sonication (3), treatment with phenylethanol (13), and treatment with toluene (6). Toluene treatment resulted in the greatest increase in enzyme activity above that observed with untreated cells (110 versus 15% increase for sonication, >95% decrease for cold shock, and 70% decrease for phenylethanol). The toluene procedure was performed as follows: cell preparations were initially suspended and subdivided into aliquots of approximately 10 ml each since toluene vaporization was more effective when carried out in smaller volumes. A volume of toluene equal to 5% of the aliquot volume was added, and the suspension was vigorously mixed for 20 s. The aliquots were then combined, and the phases were allowed to separate. Toluene was
TABLE 1. Effect of experimental conditions on thiocyanate production

<table>
<thead>
<tr>
<th>Experimental condition/treatment</th>
<th>μmol of SCN⁻ formed per min</th>
<th>Reaction(s) occurring&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.059</td>
<td>i, ii, iii</td>
</tr>
<tr>
<td>Extract boiled</td>
<td>0.024</td>
<td>ii, iii</td>
</tr>
<tr>
<td>Extract boiled; S₂O₃⁻ omitted</td>
<td>0.003</td>
<td>iii</td>
</tr>
<tr>
<td>Extract boiled; S₂O₃⁻ omitted; Formalin added before CN⁻</td>
<td>&lt;0.001</td>
<td>None</td>
</tr>
<tr>
<td>Unboiled; S₂O₃⁻ omitted; Formalin added before CN⁻</td>
<td>&lt;0.001</td>
<td>None</td>
</tr>
</tbody>
</table>

<sup>a</sup> All assays were performed in triplicate for 5 min with toluene-permeabilized cell suspensions.
<sup>b</sup> Refers to reactions described in the text.

removed with a pipette, and the aqueous phase was assayed for rhodanese activity.

A boiled enzyme control was instituted to account for nonenzymatic thiocyanate production. A sample of the toluene-treated cell suspension was boiled for 30 min to denature cellular proteins, cooled, and assayed for rhodanese activity.

Table 1 presents the relative contributions of the three thiocyanate-producing reactions described above. As indicated, the control produced thiocyanate from all three reactions. Boiling eliminated the rhodanese-catalyzed reaction; consequently, the second condition indicates the combined contribution of the second and third reactions. The third experimental condition reflects the contribution of only the third reaction. The fourth and fifth conditions demonstrate that there was no production of thiocyanate when formaldehyde was added, either with or without active enzyme.

The nonenzymatic reactions were clearly significant relative to the enzymatic reaction. The spontaneous nonenzymatic reaction of thiosulfate and cyanide at 50 mM concentration of reactants occurred linearly at a rate of 0.021 μmol/min, or approximately one-half of the enzymatically catalyzed reaction. Calculating from the constant for the reaction given by Davis (5) for nonmarine conditions at 25°C gives a rate of 0.015 μmol/min, in good agreement with our experimental result. The rapid spontaneous cyanalysis of elemental sulfur (1) in the permeabilized cell pellet occurred at a rate about one-tenth of the enzyme-catalyzed reaction. Elemental sulfur is an unavoidable by-product of Thiobacillus metabolism of thiosulfate in culture (9).

It was clear that a method was needed to distinguish between the nonenzymatically produced thiocyanate and the enzymatically produced thiocyanate. We used the boiled control for each sample as the instrument blank for that sample. Therefore, the reactions in this control accounted for all nonenzymatic thiocyanate production for each particular sample. Consequently, rhodanese activity of each sample was directly indicated by the spectrophotometer since the nonenzymatic thiocyanate production had been instrumentally corrected.

Previous investigations have assayed rhodanese by using as a spectrophotometer blank a sample to which formaldehyde was added before the cyanide substrate (3, 15, 18) under the assumption that formaldehyde was denaturing the enzyme. However, formaldehyde will also quickly remove any cyanide from the reaction mixture by nonbiological chemical reactions (10). Therefore, since all three reactions require cyanide to produce thiocyanate, the use of a mixture to which formaldehyde was added before cyanide as a blank is inappropriate since it eliminates both the enzymatically catalyzed reaction and the nonenzymatic thiocyanate-producing reactions. Our instrument blank removed only the enzymatically catalyzed reaction.

To make meaningful conclusions concerning the physiological activity of the enzyme, accurate measurements of enzyme activity are essential. Previous applications of the thiocyanate assay for rhodanese must be questioned in that they did not account for the significant amount of nonenzymatic thiocyanate production. Institution of the boiled control as an instrument blank takes nonenzymatic thiocyanate production into account, and gives a value which reflects only the thiocyanate produced by the rhodanese-catalyzed reaction.

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