Mechanism of Rhodanese Catalysis of Thiosulfate-Lipoate Oxidation-Reduction*

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(Received for publication, July 15, 1963)

Crystalline beef liver rhodanese (thiosulfate-cyanidesulfur-transferase, EC 2.8.1.1) catalyzes sulfur transfer from SSO₄²⁻ to CN⁻ by a double displacement mechanism (3, 4). The biological role of this reaction is not clear, but its possible involvement in CN⁻ detoxiation has been repeatedly stressed. The present study of the specificity of the crystalline beef liver enzyme and of the occurrence of the enzyme in some common microorganisms has yielded a better understanding of the physiological role of rhodanese. Rhodanese was found to catalyze the reduction of SSO₄²⁻ to HS⁻ and SO₄²⁻ when lipoic acid or lipoamide was used as reducing agent (1). The mechanism of rhodanese-catalyzed SSO₄²⁻ reduction was studied, and an intermediate in the reduction was identified as an activated form of sulfide (2).

EXPERIMENTAL PROCEDURE

Crystalline beef liver rhodanese was prepared by the method previously described (5). dl-Lipoic acid was purchased from the California Corporation for Biochemical Research. dl-Lipoamide was purchased from the Aldrich Chemical Company. Benzenethiosulfonate was synthesized by a modification of the method of Traeger and Linde (6). Cysteine-S-sulfonate was synthesized by the method of Kolthoff and Strickes (7) and purified on a column of Dowex 1 in the NO₃⁻ form. dl-Dihydrolipoate was prepared immediately before use in the enzymic assay by NaBH₄ reduction of dl-lipoic acid under N₂ in a buffer containing sodium phosphate and sodium tetraborate, both at 0.05 M, pH 8.5. The dl-dihydrolipoate used in nonenzymic reactions was prepared by NaBH₄ reduction in 0.1 n NaOH. Spectrophotometric measurements were performed with a Perkin-Elmer model 202 recording spectrophotometer.

The use of a Heyrovsky recording polarograph with a dropping mercury electrode permitted simultaneous measurement of the concentrations and half-wave potentials of lip(SH)₂, HS⁻, SSO₄²⁻, and lip₂S⁻ at the pH of the enzyme assay, as previously reported (1). In 0.1 n NaOH, the half-wave potentials of these substances and lipoate persulfide were shifted toward more negative potentials by less than 0.1 volt. Reactions involving stoichiometric amounts of enzyme were carried out in a polarograph microcell with a 0.1-ml sample volume.

Bacillus subtilis and Escherichia coli were grown on a minimal medium containing salts, glucose, and citrate (8). Bacillus steaothermophilus (ATCC 7054) and Bacillus coagulans (ATCC 12245) were grown on this medium enriched with 0.2% casein hydrolysate and vitamins including thiamine, riboflavine, pantothenate, niacin, pyridoxine, inositol, and choline. Culture flasks containing liquid media were shaken for vigorous aeration. Cells in stationary phase were harvested by centrifugation, washed in 0.85% NaCl, and suspended at a density of 25 to 30 mg, dry weight, per ml in a buffer consisting of acetate and glycine, both at 0.1 M, pH 5.0. Cell suspensions were subjected to sonic disruption in an MSE ultrasonic disintegrator, model 60W, for 15 to 20 minutes or until all cells were broken. Rhodanese activity was determined by the method of Sörbo (9), modified to increase the sensitivity for small amounts of enzyme. The assay was conducted in a 1.0-ml volume for 3 minutes at 37°C. Neurospora crassa was obtained as a lyophilized powder from Worthington Biochemical Corporation, suspended in the acetate-glycine buffer, and extracted by sonic disruption for 30 minutes.

RESULTS

Specificity of Rhodanese for Sulfur Acceptor—Crystalline beef liver rhodanese accepts sulfur from SSO₄²⁻ and organic thiosulfonates (10). This reaction involves cleavage of the sulfur-sulfur bond and formation of an enzyme-sulfur compound. The sulfur of the enzyme-sulfur compound then reacts with a second substrate to form a product and regenerate the free enzyme (3, 4).

The specificity of the reaction of the enzyme-sulfur compound with the second substrate was studied to determine whether reactions other than sulfur transfer to CN⁻ were possible. A number of reducing agents were found to serve as substrates in a rhodanese-catalyzed reductive dismutation of SSO₄²⁻ to HS⁻ and SO₄²⁻. Of the physiological electron donors tested, only dihydrodipicolinic acid and dihydrodipicolinamide were active, although NaBH₄, Na₂S₂O₄, and 2,3-dimercaptopropanol could also act as substrates for the enzyme-catalyzed reduction. Cysteine, DPNH, TPNH, GSH, and mercaptoethanol were completely ineffective as reducing agents at concentrations up to 0.01 M. Regardless of the concentration of thiosulfate or enzyme, utilization of added dl-lip(SH)₂ never exceeded 50%, indicating that the enzymic reaction is stereospecific (1).

The stoichiometry of rhodanese-catalyzed SSO₄²⁻ reduction was studied by polarographic techniques. The simultaneous measurement of the disappearance of SSO₄²⁻ and the appearance of lip₂S⁻ and HS⁻ as previously reported (1) established the following reaction.

$$\text{SSO}_4^{2-} + \text{lip(SH)}_2 \rightarrow \text{HS}^- + \text{lip}_2\text{S}^- + \text{SO}_4^{2-} + \text{H}^+$$
Utilization of the active isomer of lip(SH)\textsubscript{Z} approached 100\%, indicating that the equilibrium lies far to the right (1). The reverse reaction, starting with SO\textsubscript{3}\textsuperscript{2-}, HS\textsuperscript{-}, lipS\textsubscript{Z}, and enzyme, could be detected, but the amount of SSO\textsubscript{3}\textsuperscript{-} formed at equilibrium was too small to be measured accurately. When the lipS\textsubscript{Z} and sulfide were mixed, however, they reacted spontaneously to form a chromophoric group. Fig. 1 shows the negligible absorption of sulfide at wave lengths greater than 300 \textmu m, the weak absorption of lipoate due to the dithiolane ring, and the enhanced absorption obtained on mixing the two, with a maximum at 335 to 340 \textmu m. The following section presents evidence relating to the identity of the chromophoric group. Later sections report data which implicate the chromophoric compound as an intermediate in SSO\textsubscript{3}\textsuperscript{-} reduction.

Lipoate Persulfide—The chemical identity of the chromophore was investigated in model systems in 0.1 \textit{N} NaOH, which favored its stability. Cystine reacted with S\textsuperscript{-} to form an analogous chromophore. As shown in Fig. 2, neither cystine nor S\textsuperscript{-} absorbs at wave lengths greater than 300 \textmu m, but in a mixture of the two, a substance was formed with an absorption maximum at 335 to 340 \textmu m.

Rao and Gorin (11) have identified the product of the reaction between cystine and S\textsuperscript{-} as an organic persulfide of the type RSSH. The absorption spectra of organic persulfides are characterized by the broad absorption maximum at 335 to 340 \textmu m. This property also serves to distinguish the organic persulfides from inorganic di- and polysulfides. Fig. 3 shows the spectrum of a mixture of inorganic polysulfides, consisting mainly of disulfide. The absorption spectrum with the shoulder at 300 \textmu m is characteristic of inorganic polysulfides; only the magnitude of the absorbance depends on the number of sulfur-sulfur bonds present. The difference in spectra eliminates the possibility that the chromophore in the lipoate system is an inorganic polysulfide chain.

The organic persulfides could also be characterized polarographically. Both the persulfide derived from cysteine and the chromophoric compound derived from lipoate displayed well-defined cathodic diffusion currents at -0.67 volt at pH 8.5. The sulfur-containing functional groups of the two compounds were also polarographically identical in alkali. Neither cysteine persulfide nor the chromophoric compound obtained from lipoate could be isolated from the mixture of disulfide compound and S\textsuperscript{-} in which they were formed, for they are in mobile equilibrium with these reactants.

The spectrophotometric and polarographic data indicate that the chromophoric compound formed from lipS\textsubscript{Z} and sulfide is

\* All potentials quoted are relative to the saturated calomel electrode as reference.
Technical protein with no augmented absorption at 335 to 340 nm.

A chromophore containing bound substrate sulfur is that of a persulfide linkage. However, the absorption spectrum of rhodanese has been shown to contain bound substrate sulfur (3, 4), but no sulfide could be detected polarographically or spectrophotometrically, as shown in Fig. 4. This behavior reflects the high reactivity expected of persulfides on the basis of Szczepkowski’s work with cysteine persulfide (12).

Persulfides as Intermediates—Thiosulfate does not react spontaneously with lip(SH)₂ under any conditions studied. In order to study the mechanism of this reaction, a model system was used in which SSOr was replaced by its more reactive analogue, benzenethiosulfonate. Benzenethiosulfonate reacted spontaneously in alkali with various sulfhydryl compounds, including cysteine, lip(SH)₂, and mercaptoethanol, to yield sulfide, the corresponding disulfide compounds, and a sulfite analogue.

NaSSO₄φ + 2 RSH ⇌ HS⁻ + RSSR + NaSO₄φ + H⁺

In the course of one of these reactions, a chromophore was formed having the same spectral characteristics as the organic persulfides formed in the back-reactions between S⁻ and the disulfide compounds. Kinetic measurements of Reaction 1 indicated that the chromophore was an intermediate in the reduction rather than a further product derived from HS⁻ and RSSR. In the reaction between benzenethiosulfonate and cysteine, for example, no sulfide could be detected polarographically until more than half of the steady state concentration of persulfide, measured polarographically or spectrophotometrically, had been formed. These observations indicate that organic persulfides are intermediates in the reduction of the thiosulfonates by thiol compounds.

Enzyme-Sulfur Compound—Crystalline beef liver rhodanese has been shown to contain bound substrate sulfur (3, 4), but no covalently bound lipote. It has been suggested by Sörbo (13) that the sulfur transferred to the enzyme from SSOr may be present in a persulfide linkage. However, the absorption spectrum of rhodanese containing bound substrate sulfur is that of a technical protein with no augmented absorption at 335 to 340 nm. Some additional absorption would be expected at these wave lengths if a persulfide linkage were present. Furthermore, persulfides decompose spontaneously in acid to form sulfide. In contrast, the enzyme-sulfur compound, like thiosulfate and the thiosulfonates, decomposes in acid to form colloidal elemental sulfur (4).

If the analogy between benzenethiosulfonate and the enzyme-sulfur compound is extended to the reductive reaction with lip(SH)₂, the enzyme-catalyzed reaction would be expected to involve a persulfide intermediate. Such an intermediate could not be demonstrated spectrophotometrically in the rhodanese-catalyzed reduction, probably because of the instability of persulfides at pH 8.6. However, it was possible to demonstrate a direct interaction of rhodanese with lipote persulfide in a reverse reaction. When CN⁻ was added to a solution containing lipote persulfide formed in a mixture of high concentrations of HS⁻ and lip₂ at pH 8.6, no SCN⁻ was formed. When rhodanese was added to this system, it catalyzed the rapid formation of SCN⁻. This evidence indicates that rhodanese can accept sulfur from lipote persulfide and transfer it to a suitable acceptor.

It was important to determine whether the enzyme responsible for the thiosulfate-lipote oxidation-reduction reaction was actually rhodanese rather than some trace contaminant protein in the enzyme preparation. Crystalline beef liver rhodanese, which sedimented as a single symmetrical boundary in the analytical ultracentrifuge (5) and migrated as a single band on analytical zone electrophoresis (14), was introduced into a polarograph cell containing lip(SH)₂. With limiting amounts of enzyme-sulfur compound, the amounts of lip₂ and HS⁻ formed were equivalent to the amount of enzyme-sulfur compound added.

E⁻S₁ + 2 lip(SH)₂ ⇌ 2 lip₂ + 2 HS⁻ + 2 H⁺ + E

This result demonstrated that the reaction of lip(SH)₂ with rhodanese-bound sulfur is the cause of sulfide formation in this system.

Specificity of Rhodanese for Sulfur Donor—Another aspect of rhodanese specificity concerned the possibility that a thiosulfate analogue in which the transferable sulfur was substituted might serve as a substrate. Such a compound is cysteine-S-sulfonate (RSSO₄⁻). Data from studies with nutritional mutants of Aspergillus nidulans have been interpreted to indicate that this compound is a direct precursor of cysteine in that organism (15). Cysteine-S-sulfonate was examined polarographically in a phosphate-borate buffer, pH 8.6, and found to display a well defined cathodic diffusion current at about −1.4 volts. When cysteine-S-sulfonate was mixed with stoichiometric amounts of enzyme in the polarograph, no reaction was observed. The same result was obtained when CN⁻ was present as a sulfur acceptor. Had rhodanese been capable of attacking the S–S bond in cysteine-S-sulfonate, the polarographic wave of the substrate would have diminished or disappeared when stoichiometric amounts of enzyme were added. It was concluded that cysteine-S-sulfonate is not a substrate for rhodanese.

In the course of these polarographic studies, it was noticed that cysteine-S-sulfonate is unstable in the presence of cysteine at pH 8.5. It rapidly decomposes, consuming 1 mole of cysteine to form cystine in a reversal of the synthetic reaction.

RSSO₄⁻ + RSH ⇌ RSSR + SO₄²⁻ + H⁺

(2)
oxidative decarboxylation of pyruvate and α-ketoglutarate (16).

...reaction is the same role that it is known to play in the...nucleus, CH₃-CH=CH-(CH₃)₂-COOH.

FIG. 5. Proposed reaction mechanism for rhodanese-catalyzed thiosulfate-lipoate oxidation-reduction. L refers to the carbon skeleton, CH₃-CH=CH-(CH₃)₂-COOH.

FIG. 6. Comparison of the roles of lipoate in the oxidative decarboxylation of pyruvate and the reduction of thiosulfate.
synthesis only via reduction to HS⁻. The data do not, however, preclude either the existence of a persulfide intermediate in SSOS⁻ reduction or the direct participation of such an intermediate in cysteine biosynthesis. Moreover, data suggesting this role for the persulfide intermediate have recently been obtained in a system from rat liver mitochondria. In this system thiosulfate sulfur is incorporated into cysteine without participation of sulfide as an intermediate.

SUMMARY

The specificity of crystalline beef liver rhodanese and the occurrence of the enzyme in some microorganisms were studied. Rhodanese was found to catalyze SSOS⁻ reduction to HS⁻ and SO₄⁻ when dihydrolipoic acid or dihydrolipoamide was used as reducing agent. The reaction with lipoate or lipoamide is apparently stereospecific. During the course of the reaction, sulfur is transferred from SSOS⁻ to rhodanese, forming a rhodanese-sulfur compound with the elimination of SO₄⁻. The rhodanese-sulfur compound then reacts with dihydrolipoate, transferring the sulfur to the lipoate to form an intermediate which has been called lipoate persulfide. Lipoate persulfide has been suggested as an intermediate in biosynthetic reactions requiring reduced sulfur.

4 J. F. Schneider and J. Westley, manuscript in preparation.

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