CURRENT MICROBIOLOGY, Vol. 3 (1979), pp. 117–120

**Current Microbiology**
An International Journal

# A High-Potential Nonheme Iron Protein (HiPIP)–Linked, Thiosulfate-Oxidizing Enzyme Derived from *Chromatium vinosum*

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**Abstract.** A thiosulfate-oxidizing enzyme was partially purified from *Chromatium vinosum*, and some of its properties were studied. The enzyme rapidly reduced HiPIP (high-potential nonheme iron protein) in the presence of thiosulfate. Cytochromes *c* of yeast and tuna and ferricyanide also acted well as electron acceptors for the enzyme; horse cytochrome *c* was a poor electron acceptor. Cytochrome *c*-552, cytochrome *c'*, and cytochrome *c*-553 did not act as electron acceptors. The enzyme was inhibited by cyanide and sulfite. On the basis of the stoichiometry in reduction of ferricyanide catalyzed by the enzyme in the presence of thiosulfate, the oxidized product of thiosulfate was inferred to be tetrathionate.

*Chromatium vinosum* acquires the energy necessary for its life processes by photosynthetic oxidation of sulfide and thiosulfate [16]. Therefore, photosynthetic oxidation pathways of sulfide and thiosulfate should occur in the organism. Our previous studies have revealed that cytochrome *c*-552 of the organism acts as sulfide-cytochrome *c* reductase and probably participates in the photosynthetic oxidation of the salt in the organism [9,12,17].

As for the oxidation of thiosulfate in *Chromatium*, three mechanisms have been reported: (i) reductive cleavage [11], (ii) cleavage by an enzyme having the rhodanese activity [15], and (iii) oxidation by withdrawing electrons [14]. However, details of the enzymatic mechanisms have been unknown in each case. Further, our attempts to find the rhodanese activity in the cell-free extracts of *C. vinosum* have so far been unsuccessful.

Previously, we had purified from *Chlorobium limicola* f. *thiosulfatophilum* a thiosulfate-oxidizing enzyme, classified in the third group mentioned above [12]. Since *Chlorobium* and *Chromatium* physiologically resemble each other [16], it could be expected that such a thiosulfate-oxidizing enzyme would participate in thiosulfate oxidation in *C. vinosum* also.

In the present investigation, we partially purified a thiosulfate-oxidizing enzyme and studied its reactions with various electron acceptors. We found that HiPIP (high-potential nonheme iron protein) of the organism acts as a good electron acceptor for the enzyme in thiosulfate oxidation.

## Materials and Methods

**Special reagents.** Cytochrome *c*-552 [3], cytochrome *c'* [3], cytochrome *c*-553 (550) [6], and HiPIP [1] were purified by the methods established by Bartsch and his co-workers. Tuna cytochrome *c* was purified by the method of Hagihara et al. [10], and yeast (*Saccharomyces oviformis*) cytochrome *c* was kindly supplied by Sankyo Co., Ltd. (Tokyo, Japan).

**Cultures.** A strain of *Chromatium vinosum* was kindly supplied by R. G. Bartsch and T. Meyer (University of California, San Diego) and large-scale cultivation of the organism was performed as described by Bartsch and Kamen [3]. Cells were harvested after growth for 5 days, and stored at −20°C before use.

**Polyacrylamide gel electrophoresis.** The electrophoresis was performed with slab gel prepared from 7.5% acrylamide in the absence of sodium dodecyl sulfate. After the electrophoresis had been carried out for 2 h at 20 mA and 150 V, the slab gel was cut into three strips, and these were separately stained by Coomassie brilliant blue, by the heme-staining reagents [5], and by the use of ferricyanide reduction activity [13].

**Enzyme assay.** The standard reaction mixture contained 10 mM phosphate buffer, pH 6.2, 0.33 mM $Na_2S_2O_3$, 10–35 $\mu$M electron acceptor, and 22 $\mu$g enzyme in a total volume of 3.0 ml. When ferricyanide was used as the electron acceptor, its concentration was 0.3 mM. After the enzyme was added to the reaction mixture, the reduction of the electron acceptors was followed spectrophotometrically with time.

**Enzyme preparation.** The cells (about 100 g in wet weight) were suspended in 200 ml of 10 mM Tris-hydrochloride buffer, pH 8.5. The resulting suspension was homogenized and treated with a sonic oscillator (20 kHz, 500 W; Blackstone, Jamestown, New York). After standing for 1 h, the suspension was centrifuged at 10,000 × *g* for 30 min to remove cell debris. The supernatant ob-

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0343-8651/79/0003-0117 $01.00

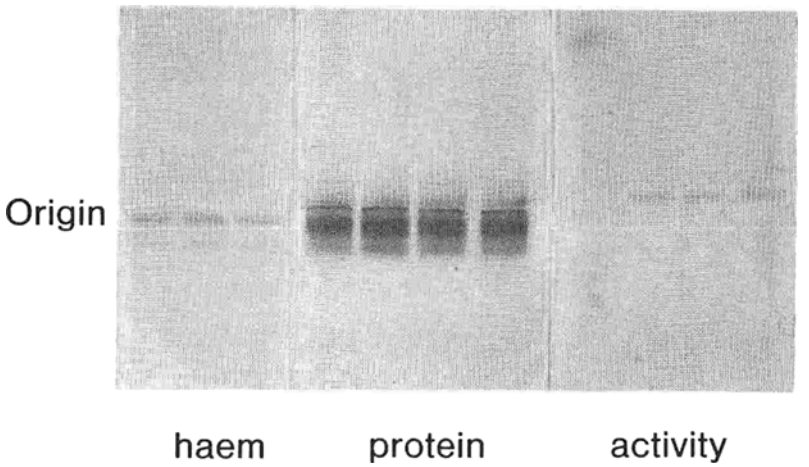


Fig. 1. Acrylamide gel electrophoretic profiles of the thiosulfate-oxidizing enzyme preparation obtained from *Chromatium vinosum*. The gel was prepared from 7.5% acrylamide. The electrophoresis was performed in 0.1 M Tris-hydrochloride buffer, pH 8.5, for 2 h at 20 mA and 150 V, and at 4°C.

tained here was centrifuged at 100,000 × *g* for 60 min, and the resulting supernatant was dialyzed for 2 days against 10 mM Tris-hydrochloride buffer, pH 8.5, with several changes of the outer solution. The dialyzed extract was fractionated with $(NH_4)_2SO_4$, and the precipitate formed between 30% and 50% saturation was collected by centrifugation at 10,000 × *g* for 30 min and dissolved in a minimal volume of 10 mM Tris-hydrochloride buffer, pH 8.5. The resulting solution was dialyzed against 0.1 M Tris-hydrochloride buffer, pH 8.5, and charged on a DEAE-cellulose column which had been equilibrated with the same buffer used for the dialysis. The enzyme was adsorbed on the column. After the column had been washed with 0.1 M Tris-hydrochloride buffer, pH 8.5, the enzyme was eluted by the linear gradient solution that was produced from 300 ml each of 0.1 M Tris-hydrochloride buffer, pH 8.5, and 0.1 M Tris-hydrochloride buffer, pH 8.5, containing 0.5 M NaCl. The eluate obtained with 0.15 to 0.20 M NaCl was able to oxidize thiosulfate. By repeating the chromatography with the DEAE-cellulose column, a very active enzyme preparation was obtained, although its purity was still low as checked by polyacrylamide gel electrophoresis. The partially purified preparation was used to determine the reaction mechanisms of the enzyme.

## Results

The enzyme preparation was still crude as checked by polyacrylamide gel electrophoresis. However, the enzyme did not contain heme; when the electrophoresis was performed at pH 4.0, the enzyme moved to anode while the heme protein moved to cathode (Fig. 1).

The enzyme preparation rapidly reduced HiPIP and cytochromes *c* of yeast and tuna in the presence of $Na_2S_2O_3$. The reduction rate of HiPIP was increased with increase of phosphate concentration until the salt concentration reached about 80 mM, while that of tuna cytochrome *c* was decreased rapidly with increase of the salt concentration (Fig. 2). In the case of yeast cytochrome *c*, the reaction rate was first increased with increase of the salt concentration and then decreased with further increase of the salt concentration. Horse cytochrome *c* was a poor electron acceptor for the enzyme even in 5 mM phosphate buffer. Ferricyanide was a good electron acceptor, and the dependency of its reduction rate catalyzed by the enzyme was similar to that of the reduction rate of HiPIP (results were not shown). Cytochrome *c*-552, cytochrome *c*-553 (550), or cytochrome *c'* derived from *C. vinosum* did not act as the electron ac-

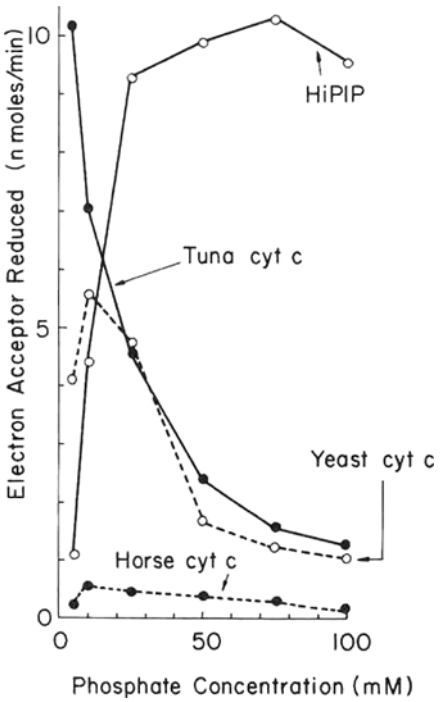


Fig. 2. Dependency on phosphate concentration of reduction rates of various electron acceptors catalyzed by *Chromatium vinosum* thiosulfate-oxidizing enzyme. The concentrations of thiosulfate, HiPIP, yeast cytochrome *c*, tuna cytochrome *c*, and horse cytochrome *c* were 330 μM, 9 μM, 10 μM and 10 μM, respectively. The amount of the enzyme preparation was 20 μg. A total volume of the reaction mixture was 1.0 ml.

Table 1. Stoichiometry in reduction of ferricyanide catalyzed by *Chromatium vinosum* thiosulfate-oxidizing enzyme in the presence of thiosulfate.[a]

| Expt no. | $Na_2S_2O_3$ added (μmol) | $K_3Fe(CN)_6$ reduced (μmol) |
|---|---|---|
| 1 | 0.400 | 0.430 |
| 2 | 0.800 | 0.810 |
| 3 | 1.20 | 1.20 |
| 4 | 1.60 | 1.58 |
| 5 | 2.00 | 1.98 |

[a] The concentration of ferricyanide was 1.5 mM, while that of thiosulfate was maximally 0.67 mM. Total volume of the reaction mixture was 3.0 ml.

ceptor for the enzyme. $K_m$ values of the enzyme were determined to be 130 μM and 3.3 mM for HiPIP and ferricyanide, respectively. The enzyme was 50% inhibited by 1.3 mM cyanide and 1.7 mM sulfite. When the enzyme was heated at 100°C for 1 min, it lost the activity completely.

Table 1 shows the stoichiometry in the reduction of ferricyanide catalyzed by the enzyme in the presence of limited amounts of thiosulfate. In this experiment, ferricyanide was added in excess of thiosulfate. The ratio of ferricyanide reduced to thiosulfate added was unity. From the results, it seems likely that thiosulfate was oxidized to tetrathionate by the enzyme.

## Discussion

The present work has revealed that in *Chromatium vinosum* a thiosulfate-oxidizing enzyme occurs which resembles the enzyme derived from *Chlorobium limicola* f. *thiosulfatophilum* [12]. One of the differences between the two enzymes is that the *Chlorobium* enzyme utilizes cytochrome *c*-555 as the electron acceptor, while the *Chromatium* enzyme does not react with cytochrome *c*-553 (550). This cytochrome is thought to correspond to *Chlorobium* cytochrome *c*-555 in a functional sense [6,12]. However, the *Chromatium* enzyme utilizes HiPIP as the electron acceptor.

The structures of HiPIP isolated from *C. vinosum* have been extensively studied [4,7]. However, its physiological role was not found although its participation in the photosynthetic electron transfer of the organism has been suggested [8]. In the present work, we have shown that the protein acts as the electron acceptor for the thiosulfate-oxidizing enzyme. HiPIP has been purified mainly from purple photosynthetic bacteria [2]. As nonsulfur purple bacteria do not utilize thiosulfate as the photosynthetic electron donor, it may be that the function of HiPIP as the electron acceptor for the thiosulfate-oxidizing enzyme is not the sole physiological role of the protein. In any case, it may be said that the present investigation has revealed at least one of the functions of the protein.

As the oxidation product of thiosulfate by the enzyme seems to be tetrathionate, it may be concluded that our present work has also confirmed the results obtained by Smith [14], with partially purified enzyme and native electron acceptor.

ACKNOWLEDGMENTS

The authors thank H. Matsubara for his encouragement during the course of this work. This work has been supported in part by Grant-in-Aid for Cooperative Research no. 334038 from the Ministry of Education, Science and Culture of Japan.

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