Physiological Studies on Thiobacilli

Part IV. Purification and Certain Properties of Cytochrome c from *Thiobacillus thiooxidans*

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Received July 20, 1967

The electrophoretically pure preparation of cytochrome c from *Thiobacillus thiooxidans* was obtained. The absorption spectrum exhibited maxima at 415, 521 and 550 m μ in reduced form. The various properties of the cytochrome were very close to those of mammalian cytochrome c, i.e., absorption spectrum, electrophoretic pattern, isoelectric point and E_0' .

During the studies of colloidal sulfur metabolism by soluble fraction of *T. thiooxidans*, it was found that an intense absorption band appeared at 550 m μ together with a faint band at 570 m μ in the soluble fraction. In recent years, a number of cytochromes from chemoautotrophic bacteria have been studied extensively. Cytochrome c of facultative anaerobe *T. denitrificans*¹⁾ and cytochromes of *T. thioparus*²⁾ were reported. Trudinger³⁾ demonstrated that four cytochromes separated from *Thiobacillus X* had the relation to thiosulfate oxidation. In this paper the comparison of cytochrome c from *T. thiooxidans* with mammalian cytochrome c was reported.

MATERIALS AND METHODS

Materials. Crystalline cytochrome c of horse heart and NADPH were purchased from Sigma Chemical Co.

Spectrophotometric determination. The spectrophotometric determination were carried out using the Hitachi model EPU-2 spectrophotometer. The spectra

of cytochromes were taken with the Shimadzu model MPS-50 recording spectrophotometer.

Ultracentrifuge runs. The sedimentation velocities were measured with the Hitachi model UCA-1 ultracentrifuge operating at 59,786 rev/min.

Disk electrophoresis. Anionic system and cationic system were prepared according to Ornstein and Davis⁴⁾ and Reisfeld, Lewis and Williams⁵⁾, respectively. The polyacrylamide gel column (0.5 $\times$ 7.5 cm) was composed of a large-pore gel (spacer gel) and a small-pore gel (running gel), the sample in 40% sucrose solution instead of sample gel was applied to the gel column. The sample was run at pH 8.6 in Tris-glycine buffer, and at pH 4.5 in β -alanine-acetate buffer for 60 min with a current of 3 mA per column. Immediately after the end of electrophoresis, the gel was stained for 1 hr with 1% amidoshwarz dissolved in 7% acetic acid and was destained by electrically in 7% acetic acid.

Paper electrophoresis. Paper electrophoresis was performed on 5 $\times$ 30 cm filter paper for 9 hr with 15 mA at 4°C using glycine-NaOH buffer (the adjustment of constant ionic strength (0.1) of buffer solution was achieved by adding sodium chloride to the buffer). The protein was detected by staining with bromphenol blue.

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2) R. Klimek, B. Skarzynski and T. W. Skarzynski, *Acta Biochim. Polon.*, **3**, 261 (1956).

3) P. A. Trudinger, *Biochim. Biophys. Acta*, **30**, 211 (1958).

4) L. Ornstein and B. J. Davis, Disk electrophoresis. Unpublished work. Printed by Distillation Products Industries, Div. Eastman Kodak Co. Rochester, N. Y.

5) R. A. Reisfeld, U. J. Lewis and D. E. Williams, *Nature*, **195**, 281 (1962).

Determination of E_0' . The measurement was carried out by the method of H. E. Davenport and R. Hill.⁶⁾ The mixtures of potassium ferricyanide and ferrocyanide were used as oxidation-reduction buffer in the solution of cytochrome c. The solution of 0.1 M phosphate buffer (pH 7.0) containing 0.01 M ferricyanide and cytochrome c was pipetted into anaerobic cuvette and the same solution as above in composition except that ferricyanide was replaced with 0.01 M ferrocyanide was pipetted into side arm of anaerobic cuvette. After the cuvette had been evacuated the tap was closed and the solution in the side arm was tipped into cuvette. After standing in ice bath for 5 min, the concentration of reduced cytochrome c was measured by the spectrophotometric method.

Purification procedure. The soluble fraction (previously reported⁷⁾ S-1) was fractionated with ammonium sulfate into three fractions precipitating between zero and 40, 40 and 70, and 70 and 100 % saturation. The pH was kept at 7.0 with ammonium hydroxide solution, during ammonium sulfate addition. Each precipitate was collected with centrifugation at $10,000 \times g$ for 10 min and dissolved in a small amount of deionized water and the solution was dialyzed overnight against deionized water at 4°C. The fraction III (40 to 70 % saturation) was applied to a column of Amberlite XE-64 (2 $\times$ 5 cm) which had been equilibrated with 0.25 M ammonium phosphate buffer (pH 7.0). After rinsing the column with 5 % saturated ammonium sulfate solution, the cytochrome adsorbed in the column was eluted with 20 % saturated ammonium sulfate solution. The eluted cytochrome was concentrated by the addition of solid ammonium sulfate to 70 % saturation. The precipitate collected by centrifugation ($10,000 \times g$ for 10 min) was dissolved in deionized water and the solution was dialyzed overnight against deionized water. The precipitate formed during the dialysis was removed off. The partially purified cytochrome was repeatedly subjected to Amberlite XE-64 column by the above mentioned procedure.

RESULTS AND DISCUSSION

Homogeneity of purified cytochrome c.

1) **Ultracentrifuge analysis.** The purified cytochrome c was analyzed in the ultracentri-

FIG. 1. Sedimentation Pattern of Purified Cytochrome C from *T. thiooxidans*.

Protein concentration was 13.3 mg/ml in 4.8 % ammonium sulfate solution. The picture was taken at the 50 min after reaching 59,730 rpm at 6°C.

FIG. 2. Electrophoretic Pattern of Purified Cytochrome c by Disk Electrophoresis.

S: Start line

A: Tris-glycine buffer, pH 8.6

B: β -Alanine-acetate buffer, pH 4.5

fuge at the concentration of 13.3 mg/ml. As shown in Fig. 1, the purified cytochrome c showed almost a single peak. The sedimenta-

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tion coefficient ($S_{20,w}$) was calculated to be 2.2 Svedberg units.

2) **Electrophoresis.** The purified cytochrome c was subjected to disk electrophoresis in polyacrylamide gel at pH 4.5 in β -alanine-acetate buffer and at pH 8.6 in Tris-glycine buffer. Basic protein should migrate toward the cathode in the 4.5-gel and acidic protein toward anode in the pH 8.6-gel. As shown in Fig. 2, in the pH 4.5-gel a single band of cytochrome c was found, however, in the pH 8.6-gel any band of protein could not be detected.

Absorption spectrum.

Fig. 3 shows the absorption spectra of purified cytochrome c of *T. thiooxidans*. The

FIG. 3. Absorption Spectra of Cytochrome c of *T. thiooxidans*.

Solid line: reduced form,
Broken line: oxidized form

absorption maxima were found at 415, 521 and 550 $m\mu$ in reduced form and at 413 and 525 $m\mu$ in oxidized form.

Electrophoresis of mammalian cytochrome c and *T. thiooxidans* cytochrome c.

As shown in Fig. 4, *T. thiooxidans* cytochrome c migrated at the same distance as mammalian cytochrome c did and the

FIG. 4. Electrophoretic Patterns of *T. thiooxidans* and Mammalian Cytochrome c by Disk Electrophoresis.

Electrophoresis was carried out at pH 4.5 in β -alanine-acetate buffer for 60 min, with 3 mA/column.

T: *T. thiooxidans* cytochrome c
M: Mammalian cytochrome c
MT: Mixture of both cytochrome c

mixture of both cytochromes migrated uniformly.

Isoelectric point.

Isoelectric point cannot be calculated exactly by paper electrophoresis because of electro-osmosis and evaporation of solvent from supporting medium during migration. However, isoelectric point of *T. thiooxidans* cytochrome c can be compared with that of mammalian cytochrome c by the paper electrophoresis. The experiments were carried out at pH 9.69, 10.18, 11.22 and 11.70, respectively, and electropherograms of the both cytochromes at various pH were transcribed on a sheet of paper. As shown in Fig. 5, electropherograms of both cytochromes showed equal patterns at pH 10.65 and 11.22. Isoelectric point calculated from electropherogram was about 10.6.

FIG. 5. Paper Electrophoretical Patterns of *T. thiooxidans* and Mammalian Cytochrome c.

Electrophoresis was performed at 4°C, for 9 hr, with glycine-NaOH buffer (ionic strength, 0.1, was adjusted by adding NaCl to the buffer). The protein was detected by staining with bromphenol blue.

Hatching area: *T. thiooxidans* cytochrome c

Dotting area: Mammalian cytochrome c

The oxidation-reduction potential.

Plots of $\log (\text{red./ox.})$ cytochrome *versus* $\log \text{Fe}^{2+}/\text{Fe}^{3+}$ are given in Fig. 6. Since E_0' for the ferrocyanide:ferricyanide system is +0.43 V at pH 7.0, the E_0' for cytochrome c is the value which from +0.43 V takes -0.06 times the logarithm of the $\text{Fe}^{2+}:\text{Fe}^{3+}$ ratio at the point where the cytochrome is half reduced, namely where $\log (\text{red./ox.})$ cytochrome c is zero. The E_0' for both cytochrome c was found to be about +0.253V.

Reduction of *T. thiooxidans* and mammalian cytochrome c.

As shown in Fig. 7, both cytochrome c preparations were reduced in a similar manner by NADPH-cytochrome c oxidoreductase partially purified from *T. thiooxidans*. It may be concluded from these results that the properties of *T. thiooxidans* cytochrome c are

FIG. 6. Oxidation-Reduction Potentials of *T. thiooxidans* and Mammalian Cytochrome c.

Effect of varying proportions in ferrocyanide-ferricyanide mixtures on the degree of reduction of cytochrome c.

FIG. 7. Reduction of *T. thiooxidans* and Mammalian Cytochrome c by NADPH-cytochrome c Oxidoreductase of *T. thiooxidans*.

Reaction mixture: phosphate buffer (pH 7.0), 100 μ moles; NADPH, 1 μ mole; cytochrome c about 0.1 μ moles; partially purified cytochrome c reductase of *T. thiooxidans*. Total volume was 3.3 ml.

very close to these of mammalian cytochrome c.

Medical School, for his help in the ultracentrifugations. Thanks are given to Mr. S. Takuma for his assistance in the experimental work.

Acknowledgement. The authors are grateful to Mr. M. Kuroda, Okayama University