Purification of Cytochrome *a* of an L-Glutamate-producing Microorganism, *Brevibacterium thiogenitalis*[†]

Yoshio SUGIYAMA, Kazuaki KITANO* and Toshihiko KANZAKI*

Takasago Plant, Takeda Chemical Industries, Ltd., Takasago, Hyogo, Japan

Received December 4, 1972

The respiratory chain system of *Brev. thiogenitalis* grown in the presence of copper ions contained cytochromes *a*, *b* and *c*. The cytochrome *a* was solubilized and purified from the cell-free extracts by means of Triton X-100 and cholate extraction, and DEAE-cellulose chromatography. It was purified about 130-fold from the cell-free extracts and was free from other cytochromes. The purified preparation contained 1.4 mμatom copper and 1.9 mμatom iron per mμmole heme *a*, respectively, and approximately 5 mμmoles heme *a* per mg protein.

It has been well established that cytochrome *a* of mammals and yeasts contains copper as well as iron.^{1~6)} Moreover, many investigators^{7~9)} observed that a specific deficiency of cytochrome *a* and a conspicuous decrease in cytochrome oxidase activity in rat liver were caused by a copper deficient diet. On the other hand, little is known about bacterial cytochrome *a*. No cytochrome *a* has yet been solubilized and purified from bacterial sources, so all data available have been obtained spectrophotometrically using whole cell suspensions or membrane fragments.^{10~12)}

In the previous paper,¹³⁾ the authors demonstrated that the formation of L-glutamate from acetate by L-glutamate-producing bacteria was markedly enhanced by the presence of copper ions in growth media. It was also noticed that succinic oxidase activity of copper sufficient cells was higher than that of deficient cells, which suggested a close relationship between copper and respiratory activity. The above and some other findings led us to study the respiratory chain of the bacteria.

This paper describes the procedure used for the purification of cytochrome *a* from

cell-free extracts and the occurrence of copper in the purified preparation.

MATERIALS AND METHODS

Organism and culture conditions. *Brevibacterium thiogenitalis* D-248 (an oleic acid-requiring mutant) was used. The composition of media and culture conditions were the same as in the fermentation with acetate described previously.¹³⁾ Copper ions were added as CuSO₄·5H₂O (0.125 μg per ml) in the main medium (acetate medium).

Analyses. Measurement of absorption spectra was performed by a Hitachi-Perkin Elmer spectrophotometer model 124, fitted with a Hitachi recorder QPD-34, using a cuvette of 1-cm light path. The concentrations of cytochromes, *a*, *b* and *c* in crude preparations were determined by the use of the difference extinction coefficients of 11.9,¹⁾ 14.4¹⁴⁾ and 21.0 mm⁻¹ cm⁻¹¹⁵⁾ at the alpha peaks in reduced minus oxidized spectra, respectively. The concentration of cytochrome *a* in purified preparations was calculated by using the difference extinction coefficient of 16.5 mm⁻¹ cm⁻¹¹⁾ between 601 and 630 mμ in the reduced state.

Total iron and copper concentrations were determined according to the method of Yonetani.³⁾ Disc electrophoresis was performed by the method according to Ornstein¹⁷⁾ and Davis.¹⁸⁾ Protein concentration was determined according to the method of Lowry *et al.*¹⁶⁾

Purification of cytochrome *a* from cell-free extracts. All the following treatments were performed at 0~4°C. The cells were harvested at a late logarithmic growth phase by centrifugation and washed twice with 0.1 M

[†] This work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, held in Sendai, April, 1972.

* Microbiological Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan.

potassium phosphate buffer (pH 7.5). Wet cells (100 g) were suspended in 1 liter of the above buffer and disrupted by a Kubota 10 kc sonic oscillator model 200 M at 160 W output for 30 min in 50 ml each batch. Triton X-100 (20 ml) was added to the solution, followed by stirring overnight. The solution was centrifuged at $10,000 \times g$ for 30 min. To the supernatant solution (940 ml), solid ammonium sulfate was slowly added to 30% saturation, and the mixture was stirred for 1 hr, followed by centrifugation at $10,000 \times g$ for 10 min. A yellow layer which appeared on the top was carefully removed. To the lower clear brown layer, solid ammonium sulfate was further added to give 60% saturation. After standing for 2 hr, the solution was centrifuged at $10,000 \times g$ for 10 min. The precipitate was collected and dissolved in 200 ml of the buffer described above. After removal of insoluble materials by centrifugation, the solution was supplemented with sodium cholate to give a concentration of 0.5% (w/v) and then ammonium sulfate was added to 20% saturation. The mixture was stirred overnight and centrifuged. To the supernatant solution ammonium sulfate was added to 30% saturation and the mixture was stirred for 2 hr. The solution was centrifuged at $10,000 \times g$ for 10 min. The resulting gelatinous sediment was collected and dissolved in 30 ml of 0.01 M Tris-HCl buffer (pH 8.0) containing Triton X-100 (0.5 w/v%), followed by dialysis against the same buffer. The dialyzed solution was applied to a DEAE-cellulose column (3×50 cm), previously equilibrated with the above buffer. A linear gradient elution was performed by a two chamber system with 0.2 M Tris-HCl buffer (pH 8.0) containing Triton X-100 in the first chamber and 2 M Tris-HCl buffer (pH 8.0) containing Triton X-100 in the second one and at a flow rate of *ca.* 30 ml per hr. Fractions (ten ml each) from tube No. 12 to 32 were combined. To 210 ml of the pooled fraction which contained cytochromes *a* and *c*, solid ammonium sulfate was slowly added to 50% saturation. By centrifugation, the upper fluffy layer was carefully collected, dissolved in a small amount of 0.01 M Tris-HCl buffer and dialyzed. The dialyzed solution was subjected to the second DEAE-cellulose chromatography as described above. To the greenish eluate (tube No. 18 to 23) which contained cytochrome *a* was added solid ammonium sulfate to give 50% saturation. The upper fluff was collected by centrifugation and dissolved in 10 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing Triton X-100 (0.5%). The clear greenish solution was applied on a Sephadex G-200 column (3×40 cm), previously equilibrated with the above buffer. Cytochrome *a* solution thus obtained was used for further analyses.

Chemicals. Sodium cholate was purchased from Sigma Chemical Co., and used without further puri-

fication. Triton X-100 was obtained from Wako Chemical Co., Tokyo. Other chemicals were of the purest grade commercially available.

RESULTS

Respiratory components

The dithionite reduced minus air oxidized difference spectra of the cell-free extracts of *Brev. thiogenitalis* grown in the presence or absence of copper ions are shown in Fig. 1. Cell-free extracts of the copper sufficient cells revealed absorption peaks at 602, 563, 552 $m\mu$ which represent the alpha peak of cytochromes *a*, *b* and *c*, respectively (A), while those of the copper deficient cells revealed absorption peaks at 563 and 630 $m\mu$ indicating the alpha peak of cytochromes *b* and *d*, respectively (B).

As shown in Fig. 2, cytochrome *a* content in the cell-free extracts was proportional to the concentration of copper ions in the growth medium up to 25 μg per litre. Thus *Brevibacterium* cytochrome *a* was expected to be a copper protein like cytochrome *a* of mammals and yeasts.

FIG. 1. Difference Spectra of *Brev. thiogenitalis* Cell-free Extracts.

Cell-free extracts in the cuvette were reduced with dithionite, while those in the reference cuvette were kept under an aerobic condition. The reaction mixture contained 45 mg of protein, 300 μmoles of potassium phosphate buffer (pH 7.5) and distilled water to 3.0 ml.

- A: Spectrum of cell-free extracts of copper sufficient cells which were grown in the acetate medium containing 0.025 μg per ml of copper ions.
- B: Spectrum of cell-free extracts of copper deficient cells which were grown in the acetate medium without added copper ions.

FIG. 2. Effect of Copper Ion Concentrations in the Acetate Medium on the Contents of Cytochromes *a* and *d*.

The relative amounts of cytochromes *a* (○) and *d* (●) were expressed as per cent of the content in cell-free extracts of copper sufficient cells and in those of copper deficient cells, respectively.

Purification

Table I shows a typical example of the results on purification of cytochrome *a* of the cell-free extracts. The results indicate that only 6% of the cytochrome *a* in the cell-free extracts were recovered in the purified preparation. However, cytochrome *a* was purified to 133-fold relative to the protein content. The content of cytochrome *a* in the final preparation was approximately 5 μmoles per mg protein, which was somewhat lower than that of animal heart or yeast preparation.^{6,19)}

The purified preparation was subjected to disc electrophoresis in polyacrylamide gel at pH 8.8 in 375 mM Tris-HCl buffer with a

FIG. 3. Disc Gel Electrophoresis of Purified Cytochrome *a* Preparation.

A disc electrophoretic pattern of the purified cytochrome *a* preparation in polyacrylamide gel (7%) at pH 8.8. Protein was stained with amido black.

current of 4 mA per tube (0.5 × 8 cm). As shown in Fig. 3, it showed a single band when stained with amido black.

Absorption spectrum

The final preparation of cytochrome *a* was a completely clear solution, but when concentrated several times after dialysis against deionized water, the solution got turbid. Therefore, the spectrum was observed in the presence of a synthetic nonionic detergent, Triton X-100 (final concentration 0.5%). As

TABLE I. SUMMARY OF PURIFICATION OF CYTOCHROME *A* FROM *Brev. thio genitalis*

Fraction	Volume (ml)	Total protein ^{a)} (mg)	Cyt. <i>a</i> ^{b)} (μmoles)	Yield (%)	Cyt. <i>a</i> (μmoles) per mg protein
Triton extract	940	12088	471	100	0.039
Ammonium sulfate 30~60% sat.	200	5641	425	90	0.075
Cholate extract	30	1047	282	60	0.269
1st. DEAE-cellulose eluate	210	108	145	31	1.34
2nd. DEAE-cellulose eluate	60	7.9	40	8	5.06
Purified cyt. <i>a</i>	28	5.6	29	6	5.18

^{a)} Protein was determined by the method of Lowry *et al.*¹⁶⁾

^{b)} The content of cytochrome *a* was determined based on the difference extinction coefficient of 11.9 mm⁻¹ cm⁻¹ at the alpha peak in reduced minus oxidized spectrum.

FIG. 4. Absorption Spectra of Purified Cytochrome *a*.

The spectrum of the oxidized form (dotted line) was obtained in the presence of $10 \mu\text{M}$ potassium ferricyanide and that of the reduced form (solid line) was measured after adding a few grains of sodium dithionite. The reaction mixture in a total volume of 3.0 ml contained approximately 1.2 mg of protein, $15 \mu\text{moles}$ of Tris-HCl buffer (pH 7.5) and 15 mg of Triton X-100.

can be seen in Fig. 4, the spectra show that the main component of the preparation was cytochrome *a*. On addition of a trace amount of sodium dithionite, the completely reduced

FIG. 5. Absorption Spectrum of Pyridineferro-hemochrome of Purified Cytochrome *a*.

For the method of preparation, see text.

form was obtained, which showed absorption maxima at 601, 520 and 442 $m\mu$. The oxidized form, obtained after the addition of $10 \mu\text{M}$ ferricyanide, showed absorption peaks at 597 and 422 $m\mu$.

Prosthetic group

The heme was extracted with acid-acetone solution (1 ml conc. HCl in 100 ml acetone), concentrated almost to dryness and trans-

TABLE II. COPPER AND IRON CONTENTS OF PURIFIED CYTOCHROME *A*

Preparation No.	Cyt. <i>a</i> ^{a)} ($m\mu\text{moles}$)	Cu ^{b)} ($m\mu\text{moles}$)	Fe ^{c)} ($m\mu\text{moles}$)	$\frac{\text{Cu}}{\text{Heme } a}$	$\frac{\text{Cu}}{\text{Fe}}$
1	30.3	45.0	63.7	1.5	0.71
2	27.0	34.0	83.0	1.3	0.41
3	35.2	45.0	88.0	1.3	0.51
Average				1.4	0.54

^{a)} The content of cytochrome *a* was calculated by using the difference extinction coefficient of $16.5 \text{ mm}^{-1} \text{ cm}^{-1}$ between 601 and 630 $m\mu$ in the reduced state.

^{b)} Copper content was determined with the zinc dibenzylthiocarbamate method after digestion with conc. H_2SO_4 .

^{c)} Iron content was determined with the *o*-phenanthroline method.

TABLE III. RELATION BETWEEN COPPER AND CYTOCHROME *A* CONTENTS DURING THE PURIFICATION PROCESS

Before analyses each fraction was subjected to gel filtration on Sephadex G-200 in order to remove contaminated copper and iron ions.

Fraction	Cytochrome ($m\mu\text{moles}$)				Cu ($m\mu\text{moles}$)	Fe ($m\mu\text{moles}$)	$\frac{\text{Cu}}{\text{Heme } a}$	$\frac{\text{Fe}}{\text{Total heme}}$
	<i>a</i>	<i>b</i>	<i>c</i>	Total				
Cholate extract	32.8	48.2	35.3	116.3	56.3	309.0	1.7	2.7
1st. EDAE-cellulose eluate	24.6	trace	6.6	31.2	35.0	85.0	1.4	2.7
Purified cyt. <i>a</i>	30.0	0	0	30.3	45.0	63.7	1.5	2.1

ferred to pyridine. It was reduced with a trace amount of sodium dithionite after adjusting the pH to 10. In this way, a clear absorption spectrum of the pyridineferro-hemochrome was obtained as shown in Fig. 5. These results indicate that cytochrome *a* purified from *Brev. thio genitalis* contained heme *a* as its prosthetic group.

Copper and iron contents

Copper and iron contents in the purified preparations are shown in Table II. Assuming that the molar extinction coefficient of *Brevibacterium* cytochrome *a* is identical to that of the beef heart cytochrome *a*, the ratio (m μ -atoms per m μ mole) of copper to heme *a* was 1.4. The atomic ratio of copper to iron was calculated to be approximately 0.54. The extinction coefficient of *Brevibacterium* cytochrome *a* based on the total copper was calculated to be approximately 11.8 mm⁻¹ cm⁻¹ which was a considerably lower value than that given for the beef heart muscle preparation (16.5 mm⁻¹ cm⁻¹).¹⁾

The behavior of cytochrome *a* and copper during the purification process is also presented in Table III. As can be seen in Table III, one mole of cytochrome *a* contained at least one atom of copper at all the purification stages.

DISCUSSION

Bacterial cytochrome *a* can be classified into two groups; one shows the reduced alpha and gamma peaks at 585~595 m μ and 435~445 m μ , respectively, and the other those at 600~605 m μ and 440~445 m μ , respectively, similar to mammalian cytochrome *a*. The former has been termed cytochrome *a*₁. The latter has been represented by the term, cytochromes *a* and *a*₃, analogous to mammalian cytochrome (*aa*₃), if the spectrochemical responses of the alpha and gamma peaks toward carbon monoxide and cyanide are similar to those of mammalian cytochrome (*aa*₃). In the presence of carbon monoxide, the alpha peak around 605 m μ and the gamma

peak around 445 m μ of reduced cytochrome *a*₃ shift to approximately 590 m μ and 430 m μ , respectively, but those of reduced cytochrome *a* are not affected. From the spectrophotometric observations, it is known that various bacteria possess cytochrome *a* of the latter type. Such examples are *Mycobacterium phlei*,²⁰⁾ *Bacillus subtilis*,²¹⁾ *Micrococcus lysodeikticus*,²²⁾ aerobically grown *Rhodopseudomonas spheroides*,²³⁾ etc. An exception was found in *Staphylococcus albus* (*Micrococcus pyogenes* var. *albus*²⁴⁾) which showed reduced bands at 604, 565 and 552 m μ . The absorption spectrum of the cytochrome *a* was not affected by carbon monoxide. Thus, this cytochrome has been classified as cytochrome *a*. A study of the aerobic *Bacillus subtilis* afforded the first example of a chemical characterization of the presence of cytochrome *a* by demonstration of extractability of a heme *a* from respiratory particles using acid-acetone.²⁵⁾ In this case, however, the cytochrome *a* was identified only as cytochrome *a*₆₀₁, with no specific comparison with the mitochondrial *aa*₃.

Brevibacterium cytochrome *a* described here was regarded as cytochrome *a* (perhaps *a* and *a*₃) by the following criteria, (1) it showed the reduced alpha and gamma bands at 601 m μ and 442 m μ , (2) heme *a* was removable from the apoprotein by extraction with acid-acetone, (3) the pyridine ferrohemochrome in alkali showed an alpha band at 587 m μ , and (4) the cytochrome *a* contained at least an equimolar amount of copper to heme *a*. The last finding is noteworthy, not only because it is the first demonstration of the occurrence of copper in bacterial cytochrome *a* (*a* and *a*₃) so far reviewed, but also because it suggests the universality of the role of copper in cytochrome *a* (*a* and *a*₃).

The details of the relation between copper and the respiratory chain system will be discussed in the following paper.

Acknowledgements. The authors wish to thank to Messrs. T. Terada, K. Takeba, Y. Tokuda and I. Nakanishi, Takasago Plant of Takeda Chemical Industries, Ltd. for continued interest and encouragement throughout the course of this work and to Dr.

M. Isono for his critical reading of this paper. Thanks are also due to Mr. K. Minami for his technical assistance.

REFERENCES

- 1) H. Matsubara, Y. Orii and K. Okunuki, *Biochem. Biophys. Acta*, **97**, 61 (1965).
- 2) Y. Orii and K. Okunuki, *J. Biochem.*, **61**, 388 (1967).
- 3) T. Yonetani, *J. Biol. Chem.*, **236**, 1680 (1961).
- 4) D. E. Griffiths and D. C. Wharton, *ibid.*, **236**, 1850 (1961).
- 5) S. Horie and M. Morrison, *ibid.*, **238**, 1855 (1963).
- 6) I. Sekuzu, H. Mizushima, S. Hirota, T. Yubisui, Y. Matsumura and K. Okunuki, *J. Biochem.*, **62**, 710 (1967).
- 7) E. Cohen and C. A. Elvehjem, *J. Biol. Chem.*, **107**, 97 (1934).
- 8) H. Yoshikawa, *J. Biochem.*, **25**, 627 (1937).
- 9) C. H. Gallagher, J. H. Judah and K. R. Ress, *Proc. Roy. Soc., London*, **B 145**, 134 (1956).
- 10) L. Smith, *Bacteriol. Rev.*, **18**, 106 (1954).
- 11) L. Smith, "The Bacteria," Vol. II, ed. by G. Gunsalus and R. Y. Stainer, Academic Press Inc., New York, N.Y., 1961, p 572.
- 12) L. Smith, N. Newton and P. Scholes, "Hemes and Hemoproteins," ed. by B. Chance, R. Estabrook and T. Yonetani, Academic Press Inc., New York, N.Y., 1966, p. 395.
- 13) T. Kanzaki, I. Nakatsui, K. Kitano, Y. Sugiyama, M. Nishio and M. Ishikawa *Agr. Biol. Chem.*, in press.
- 14) K. Ohnishi, *J. Biochem.*, **59**, 17 (1966).
- 15) B. F. van Gelder and E. C. Slater, *Biochem. Biophys. Acta*, **58**, 593 (1962).
- 16) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 17) L. Ornstein, *Ann. New York Acad. Sci.*, **121**, Art 2, 321 (1964).
- 18) B. J. Davis, *ibid.*, **121**, Art 2, 404 (1964).
- 19) T. Yubisui, *Ann. Rep. Biol. Works, Fac. Sci., Osaka Univ.*, **16**, 35 (1962).
- 20) A. Asano and A. F. Brodie, *J. Biol. Chem.*, **239**, 4280 (1965).
- 21) K. Miki and K. Okunuki, *J. Biochem.*, **66**, 831, 845 (1969).
- 22) S. Ishikawa and A. L. Lehninger, *J. Biol. Chem.*, **237**, 2401 (1962).
- 23) G. Kikuchi and Y. Motokawa, "Structure and Function of Cytochromes," ed. by K. Okunuki, M. D. Kamen and I. Sekuzu, University of Tokyo Press, Tokyo, 1968, p 174.
- 24) B. Chance, *J. Biol. Chem.*, **202**, 397 (1953).
- 25) K. Miki, I. Sekuzu and K. Okunuki, *Ann. Rep. Biol. Works, Fac. Sci., Osaka Univ.*, **15**, 33 (1967).