KOR. JOUR. MICROBIOL. September, 1986 p. 270-275 Vol. 24, No. 3

Enzymological Localization of Carbon Monoxide Dehydrogenases in *Pseudomonas carboxydovorans and Acinetobacter sp.*1

Kim, Si Wouk[1], and Young Min Kim*

Department of Biology, College of Science, Yonsei University, Seoul 120, Korea

"*Pseudomonas carboxydovorans*와 *Acinetobacter sp.* 1의 일산화탄소 산화효소의 세포내 분포에 대한 효소학적 연구"

김시욱[1] · 김영민*

연세대학교 이과대학 생물학과

Abstract: The localization of carbon monoxide dehydrogenases (CO-DHs) in *Pseudomonas carboxydovorans* and *Acinetobacter* sp. 1 was examined by comparison of the distribution of CO-oxidizing activity between soluble and particulate fractions obtained after disruption of CO-grown cells by sonic oscillation and of spheroplasts by osmotic shock. When the cells were broken by sonic oscillation, most of the CO-DH activity was recovered from soluble fractions. However, disruption by osmotic lysis of spheroplasts revealed that the enzyme activity is present in the cell membrane. The results indicate the CO-DHs in these cells are loosely attached to the cytoplasmic membrane.

Key words: *Pseudomonas carboxydovorans, Acinetobacter,* CO dehydrogenase.

Carbon monoxide dehydrogenase (CO-DH) is a key enzyme responsible for CO metabolism in carboxydobacteria which are able to grow with CO as a sole source of carbon and energy (Hegeman, 1984 ; Kim and Hegeman, 1983a; Meyer, 1985 ; Meyer and Fiebig, 1985 ; Meyer and Rohde, 1984 ; Meyer and Schlegel, 1983).

Based on composite reports of studies examining the properties of CO-DH, it appeared that the CO-DHs in several carboxydobacteria are soluble enzyme (Cho, 1983 ; Cypionka *et al.*, 1980 ; Kim and Hegeman, 1981 and 1983b; Meyer and Schlegel, 1979 and 1980 ; Zavarzin and Nozhevnikova, 1977). However, it was also speculated that the enzyme might be associated with membrane (Cypionka *et al.*, 1980 ; Kim and Hegeman, 1981 ; Schlegel and Meyer, 1981). Immunocytochemical studies using protein-A gold method have shown that CO-DH in *Pseudomonas carboxydovorans* is loosely attached *in vivo* to the inner aspect of the cytoplasmic membrane (Meyer and Rohde, 1984 ; Rohde *el al.*, 1984), supporting the assumption that CO-DH is a membrane-bound, not a soluble, enzyme.

In this study, we examined the distribution of CO-DH activity in cell fractions of *P. carboxydovorans* and *Acinetobacter* sp. 1 after disruption of the cells by sonic oscillation and of spheroplasts derived from both cells by osmotic lysis to test the localization of CO-DH in these two bacteria. Present results

[1]Present address: Microbiology Laboratory, Korea Advanced Institute of Science and Technology.
*Author to whom correspondence should be addressed.

support the idea that CO-DHs in carboxydobacteria are loosely bound to the cytoplasmic membrane.

MATERIALS AND METHODS

Organisms and cultivation

P. carboxydovorans DSM 1227 was a gift from O. Meyer, Institut für Mikrobiologie, der Universität Göttingen, FGR. *Acinetobacter* sp.1 was isolated from soils in Seoul, Korea (Cho *et al.*, 1985). Cells were grown in liquid mineral medium under a gas mixture of 30% CO-70% air at 30℃ as described in a previous report (Kim and Hegeman, 1981). CO (99.5% minimum, vol/vol) was purchased from Ulsan Gas Co.

Enzyme assay

CO-DH activity was determined photometrically by the method of Kim and Hegeman (1981) using thionin dye ($\epsilon_{595}=4.2\times10^4 M^{-1}\cdot cm^{-1}$) and anaerobic cuvette.

Preparation of cell extracts

Cells were harvested in mid-exponential growth phase, washed once with 0.05 M Tris-hydrochloride buffer (pH 7.5, standard buffer), and disrupted by sonic oscillation at 0℃ as described previously (Kim and Hegeman, 1981). The suspension was centrifuged at $10,000\times g$ for 30 min at 4℃. The supernatant fluid was referred to as crude extract. The crude extract was then sedimented at $100,000\times g$ for 90 min at 4℃. The resulting supernatant fluid and precipitate were referred to as soluble and particulate fraction, respectively.

Formation and osmotic lysis of spheroplasts

For preparing spheroplasts from CO-grown cells, cells were grown with CO in the presence of sulbenicillin (500 mg/ml, Dakeda) for 20h just prior to be harvested in the mid-exponential phase. The sulbenicillin-treated cells were washed twice with standard buffer and resuspended in 0.03 M Tris-hydrochloride (pH 8.0) containing 0.6 M sucrose plus 20 mg of lysozyme (chicken egg, Sigma) and 50 nmol (*P. carboxydovorans)* or 30 nmol (*Acinetobacter* sp. 1) of disodium EDTA (Sigma) per ml to reach an optical density of 2.0 at 600 nm. NaCl (150 mM) was then added to the suspensions under gentle stirring at 30℃ after 15 min. Microscopic observations revealed that more than 90% of the rod-shaped cells changed to spheroplasts after 3h under this condition.

Spheroplasts in the precipitates after low-speed centrifugation of the lysozyme-treated cell suspensions at $10,000\times g$ for 20 min at 4℃ were suspended in cold 15 mM Tris-hydrochloride (pH 8.0) and stirred vigorously for 30 min at 4℃. Soluble cytoplasmic proteins released by osmotic lysis of the spheroplasts were obtained in the supernatant solution after centrifugation at $6,000\times g$ for 20 min at 4℃.

Protein determination

Protein was measured by modified biuret reaction with bovine serum albumin as a standard (Gornall *et al.*, 1949). The protein contents of crude extract, particulate fraction, and spheroplast were determined by the same method after boiling the samples in 20%

Table 1. Malate dehydrogenase and CO dehydrogenase activity in fractions from cells of *Pseudomonas carboxydovorans* grown under CO after sonic oscillation

Fraction	CO-DH activity		Malte-DH activity[a]	
	μmol/mg protein/min	% Yield	μmol/mg protein/min	% Yield
Crude extract	54.2	100	18.4	100
Soluble fraction	50.0	92.2	7.2	39.1
Particulate fraction	1.2	2.2	0	

[a]Activity was measured by the method of Alefounder and Ferguson (1980).

Table 2. *Malate dehydrogenase and CO dehydrogenase activity in fractions from cells of Acintobacter sp. 1 grown with CO after sonic oscillation*

Fraction	CO-DH activity		Malate-DH activity[a]	
	μmol/mg protein/min	% Yield	μmol/mg protein/min	% Yield
Crude extract	29.6	100	18.4	100
Soluble fraction	28.0	94.5	7.2	39.1
Particulate fraction	0.5	1.5	0	

[a]Activity was measured by the method of Alefounder and Ferguson (1980).

NaOH for 10 min (Meyer and Schlegel, 1978).

RESULTS

Localization of CO-DH after sonic treatment

The distribution of CO-DH activity in cell fractions from CO-grown cells after sonic treatment is shown in Table 1 and 2. When the crude extract of *P. carboxydovorans* was fractionated by ultracentrifugation, 92.2% of the CO-DH activity remained in the soluble fraction and only 2.2% of the activity was detected from particulate fraction (Table 1). Table 2 also shows that almost all the CO-DH activity in the crude cell extract of *Acinetobacter* sp. 1 can be recovered from soluble protein fraction. Malate dehydrogenase, the soluble enzyme (Alefounder and Ferguson, 1980) used as an internal reference, showed its characteristic localization, but some of the enzyme activity was not recovered by unknown reasons.

Localization of CO-DH after osmotic lysis of spheroplasts

Figure 1 shows that most of CO-grown cells of *P. carboxydovorans* changed to spheroplasts under the experimental conditions developed by the authors. It was also observed that the experimental conditions are effective in the spheroplast formation in *Acinetobacter* sp. 1 (data not shown).

Enzyme analyses after spheroplast formation showed that there is no CO-DH activity in

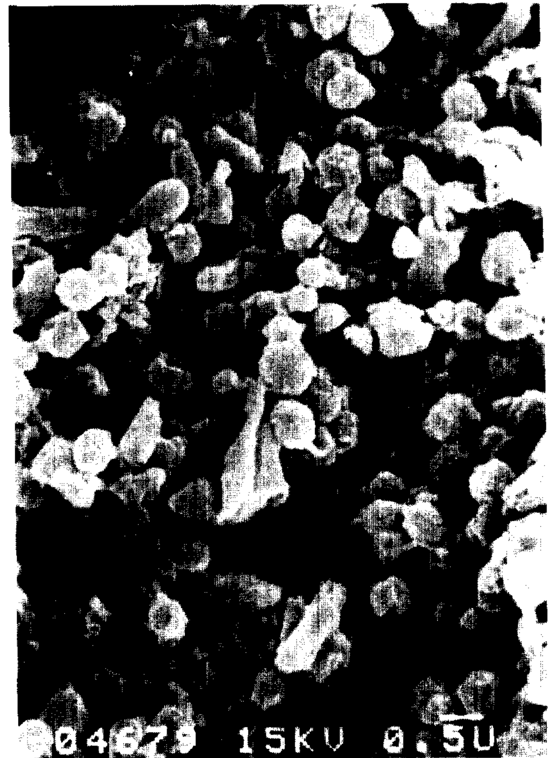

Fig. 1. *Electron micrograph of spheroplasts of P. carboxydovorans grown with CO. Cells of P. carboxydovorans treated with sulbenicillin and lysozyme were prefixed with 3% glutaraldehyde. The prefixed samples were then treated with 1% osmium tetraoxide for 2h at room temperature. After dehydration with ethanol, samples were immersed in isoamyl acetate for 30 min and dried using critical point drier (Hitachi HCP-2). The dried samples were coated with gold to 400 nm thick. A Hitachi S-450 scanning electron microscope was used for observation.*

Table 3. CO dehydrogenase activity in fractions prepared from spheroplasts of *P. carboxydovorans* and *Acinetobacter* sp. 1 grown under CO

Fraction and procedure	*P. carboxydovorans*		*Acinetobacter* sp. 1	
	Activity[a]	% Yield	Activity[a]	% Yield
Soluble periplasmic fraction	0		0	
Spheroplast	4.4	100	4.1	100
Centrifugation after osmotic shock				
Soluble cytoplasmic fraction	0		0	
Particulate fraction	4.4	100	4.1	100

[a]μmol/mg protein/min.

the soluble periplasmic fractions of *P. carboxydovorans* and *Acinetobacter* sp. 1 (Table 3). The CO-DH activity was associated only with the spheroplasts in both organisms. The data also show that all the CO-DH activity present in the spheroplasts of the two cells remained with the particulate material when the spheroplasts were lysed by osmotic shock; there was no detectable CO-DH activity in the soluble protein fractions (Table 3).

DISCUSSION

It has been generally accepted that it is very difficult to obtain protoplasts or spheroplasts from Gram-negative bacteria. Rohde *et al.* (1984) failed to obtain protoplasts from *P. carboxydovorans* when they tried to study on the localization of CO-DH in this bacterium. We, however, established appropriate conditions for spheroplast, not protoplast, formation in CO-grown cells of *P. carboxydovorans* and *Acinetobacter* sp. 1. Under the experimental conditions, over 90% of the two bacteria changed to spheroplasts. It was found that cultivation of the cells in the presence of sulbenicillin and addition of NaCl into the reaction mixture after lysozyme treatment enhance spheroplast formation in these bacteria.

Enzyme analyses after sonic treatment first showed that CO-DH activity in *P. carboxydovorans* and *Acinetobacter* sp. 1 is localized in the soluble fraction, indicating that the enzyme is soluble. However, at least 1.5%-2.2% of the CO-DH activity remained with the particulate fractions (Table 1 and 2). Since this was observed in repeated experiments, it could represent a specific membrane association of CO-DH in these bacteria. We examined this possibility using osmotic lysis of spheroplasts as a more gentle method for localization of CO-DH activity.

Enzyme analyses after spheroplast formation revealed that CO-DH activity in *P. carboxydovorans* and *Acinetobacter* sp. 1 exists in the spheroplasts exclusively, indicating that the enzyme is not a periplasmic protein. After disruption by osmotic lysis of spheroplasts, CO-DH activity was found only in the particulate material fractions, and none was detected in the soluble cytosol fractions, indicating that DO-DHs in *P. carboxydovorans* and *Acinetobacter* sp. 1 are membrane-bound, as suggested in several reports (Cypionka *et al.*, 1980; Kim and Hegeman, 1981; Meyer and Rohde, 1984; Rohde *et al.*, 1984; Schlegel and Meyer, 1981). However, the association of CO-DH with cytoplasmic membrane seems to be loose, as treatment of cells by sonic oscillation released most of the CO-DH activity into the soluble fraction (Table 1 and 2). The present work does not give any evidences whether CO-DHs in *P. carboxydovorans* and *Acinetobacter* sp. 1 are attached to the inner aspect of the cytoplasmic membrane.

Wakim and Uffen (1983) have reported that CO-DH in *Rhodopseudomonas gelatinosa*

strain 1 grown with CO anaerobically in the dark is associated with cytoplasmic membrane after an observation with spheroplasts of the cell. This, together with our results and Rohde *et al.* (1984), suggests that all the CO-DHs in both aerobic and anaerobic CO-oxidizing bacteria may be loosely attached to the cytoplasmic membrane.

적 요

*Pseudomonas carboxydovorans*와 *Acinetobacter* sp. 1에 존재하는 일산화탄소 산화효소의 세포내 분포양상을 조사하기 위하여 일산화탄소를 이용하여 성장한 세균을 초음파로 파괴하거나 세균의 spheroplast를 만들어 삼투충격으로 파괴한 후 soluble fraction과 particulate fraction에서의 일산화탄소 산화효소의 활성분포를 조사하여 비교하였다. 세균을 초음파로 파괴하였을 때는 crude cell extract에 존재하던 효소활성의 대부분이 soluble fraction에서 검출되었다. 그러나 spheroplast를 삼투충격으로 파괴하였을 때는 효소활성이 세포막부분에서만 나타났다. 이와 같은 결과는 이 세균들의 일산화탄소 산화효소가 세포막에 느슨하게 붙어 있음을 시사하고 있다.

REFERENCES

1. Alefounder, P.R., and S.J. Ferguson, 1980. The location of dissimilatory nitrate reductase and the control of dissimilatory nitrate reductase by oxygen in *Paracoccus denitrificans*. *Biochem. J.* **192**, 231-240.
2. Cho, J.W., 1983. Oxidation of carbon monoxide by carboxydobacteria isolated from soil. Master thesis, Yonsei University, Seoul, Korea.
3. Cho, J.W., H.S. Yim, and Y.M. Kim, 1985. *Acinetobacter* isolates growing with carbon monoxide. *Kor. J. Microbiol.* **23**, 1-8.
4. Cypionka, H., O. Meyer, and H.G. Schlegel, 1980. Physiological characteristics of various species of strains of carboxydobacteria. *Arch. Microbiol.* **127**, 301-307.
5. Gornall, A.G., C.J. Bardawill, and M.M. David, 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751-766.
6. Hegeman, G.D., 1984. Oxidation of carbon monoxide by bacteria. *In* "Microbial growth on C_1 compounds" (Crawford, R.L., and R.S. Hanson, eds.), pp. 21-25. American Society for Microbiology, Washington, D.C.
7. Kim, Y.M., and G.D. Hegeman, 1981. Purification and some properties of carbon monoxide dehydrogenase from *Pseudomonas carboxydohydrogena*. *J. Bacteriol.* **148**, 904-911.
8. Kim, Y.M., and G.D. Hegeman, 1983a. Oxidation of carbon monoxide by bacteria. *Intl. Rev. Cytol.* **81**, 1-32.
9. Kim, Y.M., and G.D. Hegeman, 1983b. Oxidation of carbon monoxide by *Pseudomonas carboxydohydrogena*. *Kor. J. Microbiol.* **21**, 27-35.
10. Meyer, O., 1985. Metabolism of aerobic carbon monoxide-utilizing bacteria. *In* "Microbial gas metabolism" (Poole, R.K., and C.S. Dow, eds.), pp.131-151. Academic Press, New York.
11. Meyer, O., and K. Fiebig, 1985. Enzymes oxidizing carbon monoxide. *In* "Gas enzymology" (Degn, H. *et al.*, eds.), pp.147-168. D. Reidel Pub. Co.
12. Meyer, O., and M. Rohde, 1984. Enzymology and bioenergetics of carbon monoxide-oxidizing bacteria. *In* "Microbial growth on C_1 compounds" (Crawford, R.L., and R.S. Hanson, eds.), pp. 26-33. American Society for Microbiology, Washington, D.C.
13. Meyer, O., and H.G. Schlegel, 1978. Reisolation of the carbon monoxide utilizing hydrogen bacterium *Pseudomonas carboxydovorans* (Kistner) comb. nov. *Arch. Microbiol.* **118**, 35-43.
14. Meyer, O., and H.G. Schlegel, 1979. Oxidation of carbon monoxide in cell

extracts of *Pseudomonas carboxydovorans*. *J. Bacteriol.* **137**, 811-817.
15. Meyer, O., and H.G. Schlegel, 1980. Carbon monoxide: methylene blue oxidoreductase from *Pseudomonas carboxydovorans*. *J. Bacteriol.* **141**, 74-80.
16. Meyer, O., and H.G. Schlegel, 1983. Biology of aerobic carbon monoxide-oxidizing bacteria. *Ann. Rev. Microbiol.* **37**, 277-310.
17. Rohde, M., F. Mayer, and O. Meyer, 1984. Immunocytochemical localization of carbon monoxide oxidase in *Pseudomonas carboxydovorans*. *J. Biol. Chem.* **259**, 14788-14792.
18. Schlegel, H.G., and O. Meyer, 1981. Microbial growth on carbon monoxide, formate and hydrogen: a biochemical assessment. *In* "Microbial growth on C_1 compounds" (Dalton, H., ed.), pp.105-115. Heydon, London.
19. Wakim, B.T., and R.L. Uffen, 1983. Membrane association of the carbon monoxide oxidation system in *Rhodopseudomonas gelatinosa*. *J. Bacteriol.* **153**, 571-573.
20. Zavarzin, G.A., and A.N. Nozhevnikova, 1977. Aerobic carboxydobacteria. Microbial Ecology **3**, 305-326.

(Received Feb. 19, 1986)