Induction of Hydrogenases in Non-proliferating Cells of *Alcaligenes hydrogenophilus*

Kiyohito YAGI, Masahiro URUSHIHARA, Satoshi F. OHDOH, Fusako UMEDA, and Yoshiharu MIURA*

Faculty of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565, Japan. *Received April 27, 1992*

A hydrogen bacterium, *Alcaligenes hydrogenophilus*, was grown aerobically under heterotrophic conditions. Cells were resuspended with nitrogen-free mineral salt medium and incubated under a gas mixture of H_2, O_2, and CO_2. Membrane-bound and soluble hydrogenases could be induced in the non-proliferating cells after certain lag periods. Compounds supporting rapid growth strongly repressed the hydrogenases induction in the non-proliferating cells. It was also clarified by the method that carbon dioxide affected only the induction of soluble hydrogenase. Thus, this method enables us to analyze the effect of the component, which is indispensable for growth, on the induction.

Keywords hydrogenase; hydrogen bacteria; *Alcaligenes hydrogenophilus*; hydrogen-oxidation

Hydrogen bacteria can grow using H_2 as an energy source and CO_2 as a sole carbon source. Recently, hydrogen bacteria have attracted attention because they produce biodegradable thermoplastic material, such as poly-β-hydroxybutyrate.[1] We have been trying to clone genes encoding H_2-oxidation (Hox) and CO_2-fixation ability.[2-4] Transfer of the properties into various bacteria will be a promising strategy to establish a CO_2-consuming type industrial production system. Hydrogenase is a key enzyme of Hox properties. Mechanism of the regulation is complicated and has not been fully understood. *Alcaligenes hydrogenophilus* is a gram-negative facultative hydrogen bacterium, and has been isolated from soil in this laboratory.[5] Autotrophically grown cells have membrane-bound (MH) and soluble NAD^+-reducing (SH) hydrogenases. Formation of the two hydrogenases is induced in the presence of H_2 and repressed by the addition of organic compound.[6] Since the induction has been carried out in growing cells, the effect of each component which is essential for growth, such as CO_2, could not be examined. In this work we established the hydrogenase induction system using non-proliferating cells of *A. hydrogenophilus*.

Materials and Methods

Induction The bacterium was grown aerobically at 30 °C with a half concentration of L-broth. Cells were harvested at a late-log stage of growth and suspended in nitrogen-free mineral salt medium.[5] The induction was carried out at 30 °C under a gas mixture of 80% H_2, 10% O_2, and 10% CO_2 with constant reciprocal stirring at 100 rpm. When the effect of CO_2 on the induction of hydrogenase was examined, 10% N_2 was added in place of CO_2. When the effect of organic compounds was examined, 0.2% of the compound was added to the medium.

Enzyme Assay After the induction, cells were disrupted by sonication and centrifuged for 10 min at 9500 × *g*. The supernatant was ultracentrifuged for 1 h at 105000 × *g*. The precipitate and supernatant were used for measurement of MH and SH activities, respectively. Activity of MH was assayed photometrically by measuring H_2-dependent reduction of methylene blue at 570 nm. A solution containing 0.1 ml of 3 mM methylene blue, 1 unit of glucose oxidase, 1 unit of catalase, and 2.8 ml of 50 mM phosphate buffer (pH 7.0) was saturated with H_2 in silicon rubber-stoppered cubettes at 30 °C for 10 min. The reaction was started by the injection of a membrane fraction and 0.2 μmol of glucose by syringe. The activity of SH was also assayed photometrically by measuring the H_2-dependent reduction of NAD^+ at 340 nm. A solution containing 0.1 ml of 15 mM NAD^+ and 2.8 ml of 50 mM phosphate buffer (pH 7.0) was saturated with H_2 in silicon rubber-stoppered cubettes at 30 °C for 10 min. The reaction was started by injection of the soluble fraction by syringe. The final volume was adjusted to 3 ml in both assays. One unit of activity was defined as the amount of enzyme reducing 1 μmol of the electron acceptor per min. Protein was assayed by the method of Lowry *et al.*[7] with bovine serum albumin as a standard.

Results and Discussion

Alcaligenes eutrophus, which is a hydrogen bacterium closely related to *A. hydrogenophilus*, formed hydrogenases under heterotrophic conditions. On the other hand, heterotrophically grown cells of *A. hydrogenophilus* had no hydrogenases activity. The cells were suspended in nitrogen-free mineral medium to suppress further growth and incubated under autotrophic conditions as described in Materials and Methods. Figure 1 shows the time course of hydrogenases induction. Two types of hydrogenases were induced after a certain lag period in non-proliferating cells. The addition of 100 μg/ml chloramphenicol completely inhibited the induction. An intracellular amino acid pool might be used for the *de novo* synthesis of hydrogenases.

Heterotrophically grown cells were incubated under a gas mixture of 80% H_2, 10% O_2, and 10% N_2. As shown in Fig. 2, in the absence of CO_2 MH was induced to the normal level, but the induction of SH was inhibited to less than half of the normal level. Since the physiological role of SH is thought to reduce NAD^+ to produce an electron donor for CO_2-fixation, it is possible that CO_2 affects the induction of SH. This kind of information can not be obtained in growing culture because CO_2 is indispensable for autotrophic growth. The hydrogenases in *A. hydrogenophilus* are known to be induced only in the presence of hy-

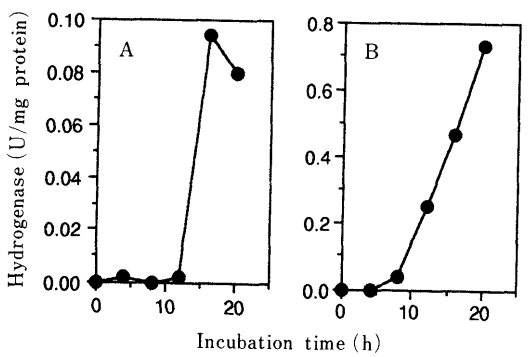

Fig. 1. Time Course of Hydrogenases Induction in Non-proliferating Cells of *A. hydrogenophilus*

Heterotrophically grown cells were incubated under a gas mixture of 80% H_2, 10% CO_2, and 10% O_2 for the time indicated. Then activities of membrane-bound (A) and soluble (B) hydrogenases were measured as described in Materials and Methods.

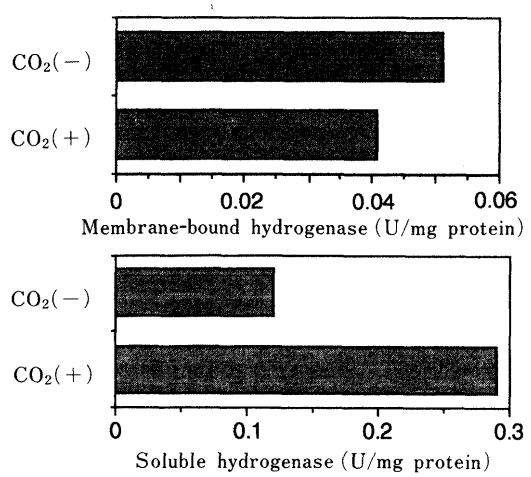

Fig. 2. Effect of CO_2 on Hydrogenases Induction in Non-proliferating Cells of *A. hydrogenophilus*

Heterotrophically grown cells were incubated under a gas mixture of 80% H_2, 10% CO_2, and 10% O_2 or mixture of 80% H_2, 10% O_2, and 10% N_2 for 20 h.

TABLE I. Effect of Organic Compounds on Induction of Hydrogenases in Non-proliferating Cells of *Alcaligenes hydrogenophilus*

Organic compound	Specific activity (U/mg protein)		Specific growth[a] rate (h^{-1})
	Membrane-bound hydrogenase	Soluble hydrogenase	
None (autotrophic)	0.28	0.96	0.25
Glycerol	0.39	1.42	0.03
D-Fructose	0.19	0.89	0.25
D-Gluconate	0.24	0.84	0.26
Acetate	0.10	0.58	0.31
Succinate	0.15	0.52	0.32

a) Bacteria were grown heterotrophically in a mineral-salt medium containing 0.5 g/l of NH_4Cl and 2 g/l of each organic compound.

drogen in growing culture.[6] Heterotrophically grown cells were incubated under a gas mixture of 80% N_2, 10% O_2, and 10% CO_2 for 20 h to examine the effect of H_2 on the induction in non-proliferating cells. Although MH activity was not detected, slight SH activity was induced using only endogenous energy reserve in the absence of H_2 (data not shown). The sensitivity to H_2 for the induction might be different between SH and MH. We have already determined the DNA sequence of MH genes and isolated the mutant strain which forms MH constitutively. It is very interesting to compare and analyse upstream regions of MH genes in wild type and mutant strains.

Organic compounds are known to repress the formation of SH and MH in growing culture of *A. hydrogenophilus*.[3,6] Table I shows the effect of various organic compounds on the induction of hydrogenases. Heterotrophically grown cells were suspended in a nitrogen-free mineral salt medium and incubated in the presence of a 0.2% organic compounds under a gas mixture of 80% H_2, 10% O_2, and 10% CO_2 for 30 h. Incubation with acetate and succinate, which supported rapid heterotrophic growth, resulted in a marked decrease in hydrogenases formation. On the other hand, the incubation with glycerol, which was a poor carbon source for the heterotrophic growth of *A. hydrogenophilus*, gave the highest level of hydrogenase induction. The regulation was independent of the growth rate because hydrogenases were induced in non-proliferating cells. The energy state in cells appears to regulate the induction. There is a large family of structurally and functionally similar two-component regulatory systems consisting of sensor and regulator proteins in bacteria.[8] Such a system was reported to be responsible for the regulation of hydrogenase gene expression in *Escherichia coli*.[9] A sensor system detecting the energy state in cells might exist and regulate the induction of hydrogenases in *A. hydrogenophilus*.

We would be able to analyse the regulation of hydrogenase expression apart from growth-related phenomenon by this method. Moreover, the method could be used to obtain a large amount of hydrogenases in a short period, especially for hydrogenase repressed by organic compounds. Cells are grown aerobically with a nutrient medium to get a large cells mass. Hydrogenase can then be induced under autotrophic conditions. Introduction of Northern blot analysis into this system will give us more detailed information about the regulation of hydrogenases.

References

1) A. J. Anderson and E. A. Dawes, *Microbiol. Rev.*, **54**, 450 (1990).
2) K. Yagi, H. Min, M. Urushihara, Y. Manabe, F. Umeda, and Y. Miura, *Biochem. Biophys. Res. Commun.*, **137**, 114 (1986).
3) K. Yagi, H. Min, T. Seto, U. Umeda, T. Doi, S. Uesugi, and Y. Miura, *J. Ferment. Bioeng.*, **68**, 225 (1989).
4) F. Umeda, N. Tanaka, N. Kimura, H. Nishie, K. Yagi, and Y. Miura, *J. Ferment. Bioeng.*, **71**, 379 (1991).
5) K. Ohi, N. Takada, S. Komemushi, M. Okazaki, and Y. Miura, *J. Gen. Appl. Microbiol.*, **25**, 53 (1979).
6) B. Friedrich, C. G. Friedrich, M. Meyer, and H. Schlegel, *J. Bacteriol.*, **158**, 331 (1984).
7) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
8) J. B. Stock, A. J. Ninfa, and A. M. Stock, *Microbiol. Rev.*, **53**, 450 (1989).
9) K. Stoker, W. N. Reijnders, L. F. Oltmann, and A. H. Stouthamer, *J. Bacteriol.*, **171**, 4448 (1989).