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The Relationship between Hydrogenase and Nitrogenase for Hydrogen Evolution in *Rhodopseudomonas sp.* KCTC 1437

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Rhodopseudomonas sp. KCTC 1437의 수소생성에 있어서의 Hydrogenase와 Nitrogenase의 관계

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Both hydrogenase and nitrogenase were found to be involved in hydrogen evolution independently in *Rhodopseudomonas* sp. KCTC 1437. The hydrogen formation in this bacterium was independent on light illumination and presence of NH_4^+. After establishment of conditions to measure the amount of hydrogen evolved by each of the enzymes *in vivo*, the several factors affecting on the hydrogen evolution, e.g. presence of gases (C_2H_2, H_2, O_2 or N_2), C/N ratio, were investigated. Hydrogenase was less inhibited than nitrogenase under O_2 and was active independent on the presence of N_2 or C_2H_2 which were the strong inhibitor of nitrogenase. Besides, the hydrogenase activity was increased after incubation with H_2. And it was verified that this bacterium consume hydrogen and photoreduce CO_2 by hydrogenase. From above results, it is concluded that hydrogenase in *Rhodopseudomonas* sp. KCTC 1437 can produce hydrogen under more favorable condition that nitrogenase.

Since photoproduction of molecular hydrogen by photosynthetic bacteria was first observed with *Rhodospirillium rubrum* growing photoheterotrophically,[1,2] the hydrogen evolution from organic compounds is generally believed to be catalyzed by nitrogenase while the utilization of hydrogen to be mediated by hydrogenase. With excess of NH_4^+, presence of O_2 and dark condition which inactivate the nitrogenase, the system responsible for hydrogen formation is ceased completely in various photosynthetic bacteria. From these results and many other studies, it was demonstrated that hydrogen evolution is closely linked nitrogen fixation and nitrogenase activity in photosynthetic bacteria.[3,4,5]

However, some photosynthetic bacteria were found to be able to evolve hydrogen gas by anaerobic fermentation in the absence of light. Recently, it was confirmed that dark fermentative growth with hydrogen production was attributed to a pyruvate: formate lyase and formate hydrogenase metabolic pathway like that in the facultative anaerobes.[6]

Hydrogenase catalyzes the reversible oxidation of the simplest molecule, H_2. However, almost all of the the hydrogenase so far examined appear to function *in vivo* to either consume or to evolve H_2; physiologically, they catalize an 'irreversible' reaction.[6,7]

In the past few years, the concerns with energy production have initiated a considerable amount of researches into the use of bacteria in solar energy conversion system to produce hydrogen which may be considered as one of the cleanest fuel resources.

This study was initiated for the purpose of hydrogen production using solar energy in large scale. Therefore, the

Key words: hydrogenase, nitrogenase, hydrogen production

studies were focused to increase the amount of hydrogen gas produced by the photosynthetic bacteria under proper conditions. The several characters of *Rhodopseudomonas* sp. KCTC 1437, the isolated photosynthetic bacteria, were already reported and the strain was suggested to produce hydrogen gas by both nitrogenase and hydrogenase.[8] In this study, it was intended to make it clear that *Rhodopseudomonas* sp. KCTC 1437 can produce hydrogen gas not only nitrogenase but also hydrogenase. And, the effects of various factors such as C/N ratio, several gases (H_2 C_2H_2, N_2 or O_2) on the productivity of hydrogen gas by both enzymes were investigated.

Materials and Methods

Organism and Culture Conditions

Rhodopseudomonas sp. KCTC (Korea Collection of Type Cultures) 1437 was grown photosynthetically in completely full test tubes of 17ml capacity, closed with caps. Culture medium was used as described by Woo, S.J. et al.,[8] And K medium (0.3% peptone, 0.3% yeast extract, 30mM glucose and 25mM $CaCl_2$; $2H_2O$) was used as complete medium with modification when necessary.[9]

Photosynthetic anaerobic cultures were incubated at 30°C under light intensity of about 6,000-10,000 lux. Aerobic cultures were carried out in shaking flask.

Preparation of Precultured Cell Suspension

Precultured cells were prepared and stored as previously described.[5,10]

Measurement of Hydrogen Evolution

Hydrogen evolution was carried out in serum bottles (40ml) containing medium to which precultured cell suspension was added. The optical density of the medium was adjusted to 0.5-0.6 at 660nm and its total volume was 10ml. The same medium like culture medium (glucose 30mM. glutamate 7mM) was used.

In order to measure the activity of nitrogenase, 10mM of hypophosphite (Hayashi pure chemical Ind. Co. Japan) which inhibited phyruvate formate lyase,[8] was added to the medium. In the case of hydrogenase, NH_4 (7mM), as the inhibitor of nitrogenase, was substituted for glutamate as N source.

Capping, gassing with argon and incubation were followed as described.[8]

After 40 hours of incubation, the amount of hydrogen produced was analyzed with a Varian 3700 gas chromatograph equipped with a thermal conductivity detector which was set at 90°C. To measure the amount of hydrogen gas exactly, Porapack Q column (80 to 100 mesh) was used at 60°C because it could detect not only H_2 but also CO_2 which was evolved in large amount at the same time during incubation. Nitrogen was used as a carrier gas.

Hydrogen content was calculated from peak heights of the recorder by reference to a calibration curve of CO_2 and H_2.

Nitrogenase Assay

Nitrogenase activity was measured by the reduction of acetylene to ethylene as described previously.[12]

Measurement of Optical Density and Determination of Cell Mass

Bacterial concentration was measured by either absorbance of culture at 660nm (using Shimazdu UV-260) or its dry weight as described.[8]

Result and Discussion

Effect of the Growth Condition

Table 1 shows the effect of various incubation conditions on the production of hydrogen gas. While nitrogenase could

Table 1. Hydrogen evolution affected by the presence of light and air.

	Amount of hydrogen produced(*ml*/vial)[a]			
	Light, aerobic[b]	Light, anaerobic	Dark, aerobic	Dark anaerobic
Hydrogenase[c]	4.83	9.99	3.52	10.03
Nitrogenase[d]	0	2.58	0	0[e]

[a]There are various units to express the amount of hydrogen evolved. The most used unit is *μl* of hydrogen/hr·mg of cell dry weight, but it is not proper in certain case that high concentration of cell produced large amount of hydrogen because the amount of hydrogen produced is divided by much amount of cell mass. Therefore, milliliter of produced hydrogen/vial was used as unit because the ultimate goal of this study is to increase hydrogen production. Each vial contains 10 *ml* medium.

[b]The condition of incubation after addition of resting cell.

[c]After nitrogenase activity was certified to be 0 by reduction of acetylene, this condition for hydrogenase activity (30 mM glucose and 7 mM NH_4Cl) was set up.

[d]After hydrogenase activity was certified to be 0, this condition for nitrogenase activity (30 mM glucose, 7 mM glutamate and 10 mM hypophosphite) was set up.

[e]Resting cells were not grown at all.

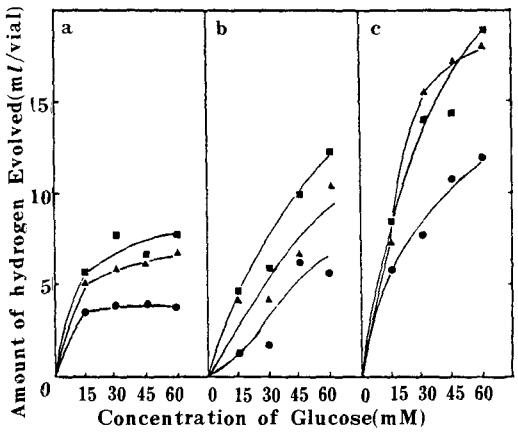

Fig. 1. Evolution of hydrogen gas at the various concentration of C and N sources.

a. Hydrogenase activity at 1mM(—●—●—), 7mM (—▲—▲—) and 21mM (—■—■—) when NH_4^+ used as N sourse.
b. Nitrogenase activity at 1mM (—●—●—), 7mM (—▲—▲—) and 21mM (—■—■—) when gluta - mate was used as N source with hypophosphite.
c. Total activity including those of hydrogenase and nitrogenase at 1mM (—●—●—), 7mM (—▲—▲—) and 21mM (—■—■—) when glutamate was used as N source.
Each vial contains 10 m*l* of medium.

evolve hydrogen gas only under light and anaerobic condition, hydrogenase could evolve it under any combined condition of light and air, even under dark, aerobic condition.

Some kinds of hydrogenase were reported to be sensitive to oxygen,[7] but the hydrogenase of this bacterium was relatively stable, although its activity was lower than that under anaerobic condition.

Besides, when resting cells grown on the complete medium were used, hydrogenase could hydrogen gas, also.

Under dark, anaerobic condition for nitrogenase, resting cells were not grown at all, because hypophosphite, the analogue of pyruvate, would inhibit the uptake and fermentation of pyruvate which was the only way to obtain energy in the condition that respiration and photosynthesis were blocked.

Effect of concentration of C and N sources

Figure 1 shows the effect of various concentration of C and N sources (Glucose was used as C source).

The higher concentration of glucose and N source were, the more hydrogen gas and cell mass were. And nitrogenase showed a severe variation as glucose concentration was increased and it is more severe in total than in nitrogenase.

It may be resulted from that nitrogenase produced hydrogen to regulate intracellular levels of ATP and reducing power obtained from large amount of C source.[13] But as N source increased at constant concentration of glucose, hydrogen production was increased, too. The result is different from that the amount of hydrogen produced by nitrogenase was reduced at high N/C ratio.[3]

It suggests that the higher cell yield which was possible at high concentration of C and N source should result in the more production of hydrogen.

Effect of Hydrogen Gas on Hydrogenase Activity

Experiments were conducted to confirm whether hydrogen, the product of this reaction, had any effects on further production of hydrogen. Resting cells were divided into 3 classs according to growth conditions that whether hydrogen existed in the gas phase and whether the hydrogenase was active.

Table 2 shows if hydrogenase is already activated in resting cells, it can have higher productivity than control. And resting cells evolved more amount of hydrogen gas when injection of hydrogen gas was carried out prior to incubation. But, it had little effect on the resting cells grown under hydrogen gas that already had active hydrogenase. Anyway, hydrogen has some effect to increase hydrogenase activity.

There were several reports to induce hydrogenase by hydrogen gas in some cyanobacteria. The cells which were grown under hydrogen gas produced hydrogen several folds according to the conditions,[14] although the major role of hydrogenase in cyanobacteria *in vivo* was the uptake of

Table 2. Relationship of hydrogenase activity and hydrogenase.

Preparation of resting cells	Amount of hydrogen produced (m*l*/vial)[a]	
	Resting cells were incubated[b] with 10% H_2	without H_2
grown at[c]		
7mM of NH_4^+[d]	10.39	7.14
7mM of NH_4^++10% H_2	8.88	9.31
control[e]	5.10	2.41

[a] Each vial contains 10 m*l* medium.
[b] The medium which composition was 7 mM of NH_4^+ and 30 mM of glucose, was used to make only hydrogenase activate.
[c] Glucose concentration was 30 mM in all the case.
[d] The growth condition for hydrogenase.
[e] The growth condition was same as the preparation of resting cells in materials and methods.

hydrogen.

In most photosynthetic bacteria, hydrogenase has two functions. One consumes hydrogen to photoreduce CO_2 and another produces hydrogen although it is not known if they are the same enzyme.

From above result, it can be assumed that hydrogenase for photoreduction induced by hydrogen gas was not to uptake hydrogen but to produce it.

In the case of nitrogenase, the evolution of hydrogen gas was also increased when incubation was carried out under hydrogen gas. The higher concentration of hydrogen existed (until 20%), the more hydrogen was produced.

Inhibition of N_2, O_2 or C_2H_2

It has been known that nitrogenase activity to produce hydrogen was inhibited by N_2, O_2 or C_2H_2. Because N_2 and C_2H_2 are one of substrates of nitrogenase, they inhibit it completely. O_2 is a strong inhibitor to introgenase and blocks hydrogen production completely. To investigate the effect of gas on hydrogenase, resting cells were incubated with various concentration of N_2, O_2 or C_2H_2 and measured the amount of hydrogen produced.

As Figure 2 shows, hydrogenase was more stable to several gases than nitrogenase generally. This fact verified our assumption again that *Rhodopseudomonas* sp. KCTC 1437 produces hydrogen gas by both enzymes. Hydrogenase has been reported to be sensitive to O_2, although the sensitivity varies with species.[7] The hydrogenase of this strain

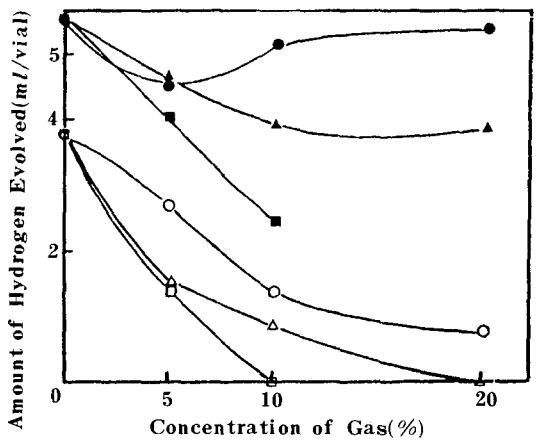

Fig. 2. Effects of several gases on hydrogen production.

Gas was injected after bubbling of argon gas. The open marks are for nitrogenase and closed ones are for hydrogenase. The circle is symbol for nitrogen gas, the triangle for acetylene gas and the square oxygen gas. Each vial contains 10 m*l* medium.

Table 3. Hydrogen uptake activity of *Rhodopseudomonas* sp. KCTC 1437[a].

Glucose concentration (mM)	Amount of hydrogen (m*l*/vial)[b]	
	with hypophosphite[c]	without hypophosphite
30	−3.7	7.4
10	−4.3	−3.1
5	−3.9	−4.0
0	−3.0	−2.9
control[d]	N. D.[e]	−1.3

[a]The medium used was composed of notified concentration of glucose and 7 mM of NH_4^+, the nitrogenase inhibitory condition, because only hydrogenase made it possible to produce or consume hydrogen. After bubbling of Ar gas, 10% of H_2 (3m*l*) was added to gas phase of vial and incubated. After determination of amount of hydrogen produced or consumed, Ar bubbling and addition of 3m*l* H_2 were done and the vial was incubated once more. The determination of amount of hydrogen existed in vial was followed by subtraction of the amount of hydrogen gas added from the measured one. The resulted data were used the amount of hydrogen gas consumed or produced.
[b]Each vial contains 10 m*l* medium. − means the consumption of hydrogen gas.
[c]Hypophosphite was used in order to make hydrogenase not to produce hydrogen.
[d]To compare to the consumption of hydrogen, control sample containing equivalent amounts of medium and hydrogen was hydrogen through leakage.
[e]not determined.

was somewhat stable to O_2. In air or 10% of oxygen, its activity reduced to 50% of the activity of anaerobic condition.

From above results, it was confirmed that the production of hydrogen mediated by hydrogenase was more convenient than by nitrogenase from the viewpoint of application.

Hydrogen Uptake Activity (HUP) of Hydrogenase

To confirm the ability to consume hydrogen for photoreduction of CO_2 by hydrogenase, the amount of hydrogen consumed during incubation was measured.

Table 3 shows this strain is able to consume hydrogen gas by hydrogenase. When cells were incubated in the low concentration of glucose which made them hardly produce hydrogen, cells consumed hydrogen gas to photoreduce CO_2. In fact, CO_2 was not detected at all when hydrogen uptake occured.

In high concentration of glucose, hydrogenase can uptake hydrogen gas when hypophosphite was included in the medium. If not, hydrogenase carried out the production of hydrogen gas instead of consumption, because the photo-

heterotrophic growth using glucose was more favorable than one through photoreduction of CO_2.

when there was no glucose, hydrogen uptake activity reduced a little compared to that in the low concentration of glucose. It was attributed to inability of the strain to grow photoautotrophically using CO_2 and H_2. In other words, photoreduction of CO_2 by H_2 was not enough for the strain to grow completely. Although some photosynthetic bacteria can grow photoautotrophically[15] with CO_2 as the sole carbon source and H_2 as the energy source, it was impossible for *Rhodopseudomonas* sp. KCTC 1437 to grow in the same manner.

It was reported that almost nitrogen fixing organism was able to consume H_2 by conventional hydrogenase. Through the system, H_2 produced by nitrogenase is directly recycled under conditions of low substrate concentration.[16] It means that the efficiency of nitrogenase is enhanced by a hydrogenase recycling electrons from substrate, lost through the hydrogen evolving function of nitrogenase.[16, 17, 18] This strain seems to use hydrogenase uptake activity for the same purpose.

It was not investigated if this was the same hydrogenase that was involved in the production of hydrogen. But, it can be assumed that it is the same enzyme which can react 'reversibly' for production or consumption of hydrogen. If it is different enzyme, the hydrogenase producing hydrogen may not activated by hydrogen, the product of this reaction.

요 약

Rhodopseudomonas sp. KCTC 1437은 NH_4^+나 빛에 무관하게 수소를 발생할 수 있다. 이 사실로 부터 이 균주는 nitrogenase 뿐만 아니라 hydrogenase 에 의해서도 수소를 생성할 수 있다는 것을 확인했다.

이 두 효소의 수소생성 능력을 ***in vivo***에서 측정할 수 있는 조건을 확립한 뒤 여러가지 조건에 대해 발생한 수소양을 측정하였다. Hydrogenase 는 nitrogenase의 수소생성을 저해하는 O_2나 N_2, C_2H_2 등에 대해서도 그 활성의 감소가 없거나 작았으며, H_2에 의해서는 오히려 수소생성능이 증대되었다. 또 이 균주는 hydrogenase에 의해 수소를 받아들여 CO_2를 광환원시킴 수도 있음도 알았다.

이상의 결과, hydrogenase가 혐기적이면서 빛이 있는 조건에서만 수소를 생성하는 nitrogenase보다 더 광범위하고 유리한 조건에서 수소를 생성할 수 있음을 확인하였다. 이러한 사실은 이 균주로 대량의 수소생산을 할 때 유리하게 이용될 수 있을 것이다.

References

1. Gest, H. and M.D. Kamen: *Science,* **109,** 558 (1949).
2. Gest, H. and M.D. Kamen: *J. Bacteriol.* **58,** 239 (1949).
3. Hillmer, P. and H. Gest: *J. Bacteriol.* **129,** 74 (1977).
4. Kim, J.S., K. Ito and H. Takahashi: *Agric. Biol. Chem.* **44,** 827 (1980).
5. Lee, J.K. and M. Bae: *Kor. J. Appl. Microbiol. Bioeng.* **11,** 211 (1983).
6. Adams, M. W.W., R.G. Upchurch and L.E. Mortenson: In "Annual Reports on Fermentative Processes," (George T. Tsao ed.), Academic Press, New York, Vol. 4, 266 (1980).
7. Adams, M.W.W., L.E. Mortenson and J.S. Chen: *Biochem. Biophys. Acta.* **594,** 105 (1981).
8. Woo, S.J., J.K. Lee, T.J. Kwon and Y.H. Kho: *Kor. J. Appl. Microbiol. Bioeng.* **13,** 257 (1985).
9. Kuhl, S.A., D.W. Nix and D.C. Yoch: *J. Bacteriol.* **156,** 737 (1983).
10. Bae, M. and J.K. Lee: *Kor. J. Microbiol.* **21,** 109 (1983).
11. Gorrell, T.E. and R.L. Uffen: *J. Bacteriol.* **131,** 533 (1977).
12. Crane, F.L., C. Windmer, R.L. Lester and Y. Hatefi: *Biochem. Biophys. Acta.* **31,** 476 (1959).
13. Gest, H.: *Adv. Microb. Physiol.* **7,** 243 (1972).
14. Tel-Or, E., L.W. Luijk and L. Packer: *FEBS Letters,* **78,** 49 (1977).
15. Madigan, M.T. and H. Gest: *J. Bacteriol.* **137,** 524 (1979).
16. Kelley, B.C., C.M. Meyer, C. Candy and P.M. Vignais: *FEBS Letters,* **81,** 281 (1977).
17. Smith, L.A., S. Hill and M.G. Yates: *Nature,* **262,** 209 (1976).
18. Dixon, R.O.D.: *Nature,* **262,** 173 (1976).