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A Study on Nitrogenase — Mediated Evolution of Molecular Hydrogen in *Rhodopseudomonas sphaeroides* K-7

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Rhodopseudomonas sphaeroides K-7 의 질소고정효소 의존성 수소생성에 관한 연구

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Rhodopseudomonas sphaeroides K-7 evolves large quantities of molecular hydrogen under anaerobic and light illuminated conditions in the presence of utilizable organic compounds as electron donors. Photoevolution of molecular hydrogen was strictly dependent on light as the activity of nitrogenase in this organism. Both of these were inhibited to the nearly same extent at varying concentrations of ammonium ion which also depressed nitrogenase synthesis. In the reaction mixtures devoid of molybdenum ion which is known as the component of nitrogenase, hydrogen evolution also decreased similarly like nitrogenase activity. Photoevolution of molecular hydrogen appeared to have no relationship with hydrogenase acitivity and bacteriocholophyll content and it was markedly inhibited under the atmosphere of C_2H_2, N_2 or O_2. The results strongly indicate that hydrogen evolution by *R. sphaeroides* K-7 might be catalyzed by nitrogenase. Both hydrogen evolution and nitrogenase activity were largely influenced by the nutritional history of the resting cells. From which we propose that glutamate might play an important role in the regulation of nigrogenase activity *in vivo*.

Photosynthetic bacteria can perform photosynthesis, nitrogen fixation and hydrogen evolution using light energy. The photoevolution of molecular hydrogen which has been now recognized as a general property of these bacteria was first observed in *Rhodospirillum rubrum* growing photoheterotrophically and anaerobically.[1] Photosynthetic bacteria are also capable of utilizing hydrogen as reductant for photoautotrophic growth at low concentrations of substrates available.[2] In the aspect of hydrogen metabolism, hydrogen evolution has been suspected to be mediated by nitrogenase, while hydrogen utilization through its uptake by hydrogenase, on the basis of many indirect evidences.[2-5] But more detailed studies on the relations between hydrogen evolution and nitrogenase still remain to pursue in regard of increasing the rate of hydrogen evolution and nitrogenase activity for possible utilizations[6,7] of the photosynthetic bacteria with practical purposes. In present communication, we aimed at elucidation of hydrogen evolution in relation to nitrogenase and their *in vivo* regulation according to nutritional history of resting cells.

Materials and Methods

Bacterial strain

Among the several strains isolated and identified as previously reported,[8] *Rhodopseudomonas sphaeroides*

K-7 was selected and used due to its excellent capacity of hydrogen evolution.

Growth media and conditions

The medium of Ormerod et al.[9] was used with slight modification. Yeast extract was omitted. Instead, thiamine (1 ug/ml) and p-aminobenzoic acid (0.2 ug/ml) were added as growth factors. To the above medium, DL-malate (30mM) was added as a electron donor (minimal malate medium). In addition, ammonium sulfate was replaced by L-glutamate (7mM) as a nitrogen source. In plate culture, 1.5% agar was added to the medium.

Cells were grown at 30°C under anaerobically illuminated condition of about 12,000 lux.

Preparation of resting cell suspensions

Resting cells were prepared by the method of kelley et al.[2] Cultures were harvested at late exponential phase and centrifuged to precipitate cells. The pellets were washed twice with 10mM potassium phosphate buffer (pH 6.8) finally resuspending in the same buffer. The resting cell suspensions were stored under argon at 0°C until required.

Measurement of hydrogen evolution

Hydrogen evolution was carried out in serum bottles (25ml) containing 10ml mixtures of resting cell suspension (A660. 0.7) and DL-malate (30mM), L-glutamate (7mM) medium. After capping the reaction vessels with gas-tight rubber stoppers, the mixtures were gassed with argon for 3 min and incubated in a shaker bath at 30°C under illuminated condition of about 12,000 lux by two 200 W incandescent lamps. After a given time passed, samples (300 ul) of the gas phase were withdrawn with a gas syringe (Precision Sampling Co.). It was analyzed for hydrogen with a Varian 3,700 gas chromatograph equipped with a thermal conductivity detector which was set at 120°C. The stainless-steel column (2m x 1/8 inch) was packed with molecular sieve 5A (45 to 60 mesh) and was maintained at 80°C. Argon was used as a carrier gas (flow rate, 40ml/min). The results were quantitated by relating the peak height to a calibration curve made with pure hydrogen obtained from hydrogen generator (General Electric Co.).

Nitrogenase assay

Nitrogenase activity was measured by the reduction of acetylene to ethylene.[10] All reaction procedures were the same as those of hydrogen evolution except that 10% of gas phase was replaced by acetylene after gassing with argon. The analysis for ethylene reduced by nitrogenase from acetylene was also with a Varian 3,700 gas chromatograph. The hydrogen flame ionization detector was used at 200°C and the stainless-steel column (1.5m x 1/8 inch) packed with Porapak R (80 to 100 mesh) was used at 80°C with nitrogen as a carrier gas (flow rate, 40ml/min). The amount of ethylene could be quantitated by relating the peak height or area to a calibration curve made with standard ethylene. Nitrogenase activity was expressed by the rate of ethylene formation.

Hydrogenase assay

Hydrogenase activity was measured by reduction of methylene blue.[11,12] Mixture (5ml) of 0.0005% methylene blue and cells (A660, 0.5) in potassium phosphate buffer was added in tube (10ml) for Spectronic 20 spectrophotometer. The gas phase was argon. Hydrogen (final 10%, v/v) was injected into the gas phase and the reduction rate of methylene blue was measured by the absorbancy decrease at 570nm. Control tube gassed with nitrogen was included to evaluate nonspecific reduction of the dye.

Measurement of bacteriochlorophyll

For bacteriochlorophyll determination, 40ml (A660, 1.0) of cell suspension was centrifuged. The pellet was extracted with 9ml of acetone-methanol (7:2, v/v), and the absorbancy of the extract was measured at 775nm. The concentration of bacteriochlorophyll was calculated using an extinction coefficient of $75mM^{-1}\ Cm^{-1}$.[13]

Cell mass determinations

Bacterial concentrations were measured either by determining culture absorbancy at 660nm or dry weight of centrifuged cells (washed once with distilled water and dried in preweighed aluminum cups at 105°C for 6 hrs).

Results and Discussion

Light dependence of hydrogen evolution and nitrogenase activity

Fig. 1 shows the effect of light on hydrogen evolution and nitrogenase activity. Under dark condition both of these did not increase, while there were consistent increases under light condition. Both of these also increased with normal rates when illuminated again. Thus, hydrogen evolution was dependent on light as nitrogenase activity. The light dependence of nitrogenase may be due to large requirement of energy needed for nitrogen fixation presumably via ATP formed by photophosphorylation.

Inhibition of hydrogen evolution and nitrogenase activity by NH_4^+ ion

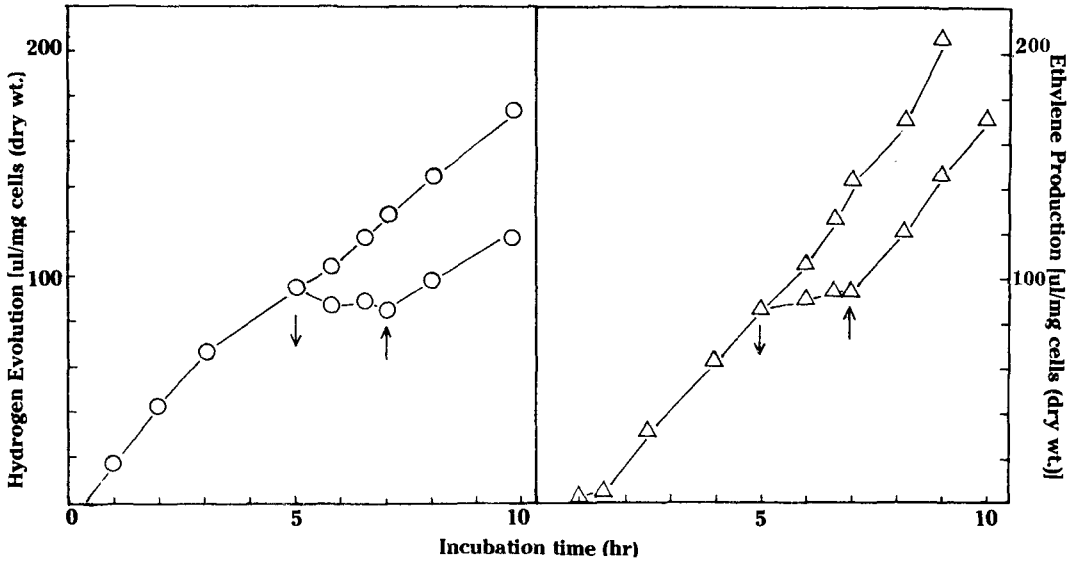

Fig. 1. Effect of Light on Hydrogen Evolution and Nitrogenase Activity.

↓: Light off, ↑: Light on, ○: Hydrogen evolution, △: Nitrogenase activity.

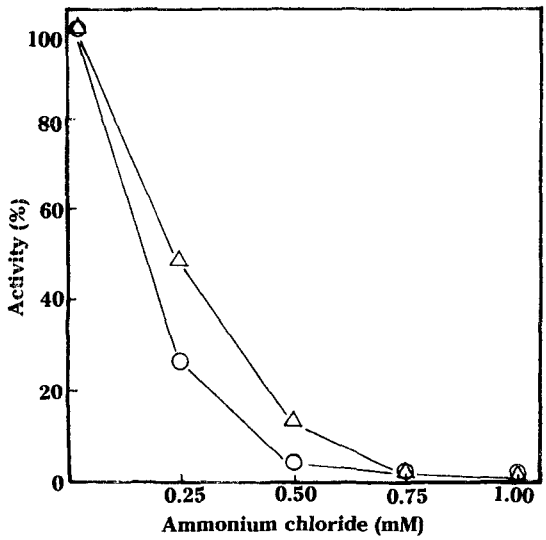

Fig. 2. Inhibition of Hydrogen Evolution and Nitrogenase Activity by Ammonium ion.

Incubation was performed for 15 hrs in minimal malate medium supplemented with NH_4Cl, and values were indicated as percentages of those in the absence of NH_4Cl. ○: Hydrogen evolution, △: Nitrogenase activity.

NH_4^+ ion, product of nitrogen fixation, is well known as an inhibitor of nitrogenase activity.[9, 14] Effect of NH_4 ion on hydrogen evolution and nitrogenase activity is illustrated in Fig. 2. Hydrogen evolution was inhibited as nitrogenase activity in a similar fashion and both of these were entirely inhibited at 1mM of NH_4^+ ion

Effect of molybdenum (Mo) ion on hydrogen evolution and nitrogenase activity

It is well known that nitrogenase requires Mo ion for its component.[15] Therefore, effect of Mo ion on hydrogen evolution was examined with nitrogenase activity under conditions of Mo-free and Mo-containing. As shown in Fig. 3, however, hydrogen evolution and nitrogenase activity gradually increased under Mo-free condition up to about 20—25hrs as those under Mo-containing condition. And then decreases of hydrogen evolution and nitrogenase activity in Mo-free mixtures were observed. This might be due to the role of Mo ion and nitrogenase already present in cells. At 20—25hrs when cultures reached the mid-exponential phase, it seemed that the Mo ion in cells became to be diluted and subsequently the synthesis of active nitrogenase was inhibited by the depletion of the ion. Consequently the decrease of hydrogen evolution and nitrogenase activity might be resulted. Thus hydrogen evolution was affected by Mo ion, component of nitrogenase.

Relations of hydrogen evolution with hydrogenase activity and bacteriochlorophyll content

Hydrogen evolution was examined during about 60 hrs with hydrogenase activity and bacteriochlorophyll content. As shown in Fig. 4, hydrogen evolution was independent of hydrogenase activity and bacteriochlorophyll content. The result agrees well with that of kelley et al.[2] They proposed that hydrogenase in the photosynthetic bacteria catalyzed

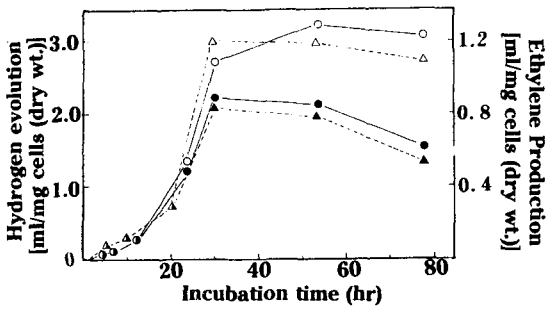

Fig. 3. Effect of Molybdenum on Hydrogen Evolution and Nitrogenase Activity.

○: +Mo, ●:-Mo) Hydrogen evolution, (△: +Mo, ▲:-Mo) Nitrogenase activity.

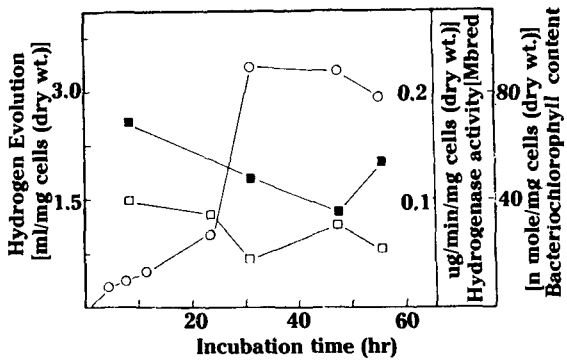

Fig. 4. Hydrogen evolution, Hydrogenase Activity and Bacteriochlorophyll Content during Incubation Period.

○: Hydrogen evolution, □: Hydrogenase activity, ■: Bacteriochlorophyll content.

uptake of hydrogen rather than evolution of it. In addition, it is evident from the result that hydrogen evolution is independent of total amount of bacteriochlorophyll, the synthesis of which was reported to be regulated by the intensity of light.[13]

From the results shown above, we can conclude that hydrogen evolution is mediated by nitrogenase.

Effect of inhibiting atmosphere on hydrogen evolution and nitrogenase activity

Hydrogen evolution and nitrogenase activity under inhibiting atmosphere are shown in Table 1. As acetylene in gas phase increased, the rates of hydrogen evolution decreased in inverse relations. This confirms that hydrogen evolution is mediated by nitrogenase. Little amount of ethylene formed under the atmosphere of 99% Ar+1%C_2H_2, which was identified as a result of insufficient amount of acetylene to be reduced by nitrogenase. At 40% Ar + 60% C_2H_2, similar value of the rate of ethylene production was obtained as that at 90% Ar + 10% C_2H_2, while at 100% C_2H_2 about 14% of the rate was inhibited which might be due to substrate inhibition as in reactions of many enzymes. An atmosphere of 100% N_2 inhibited hydrogen evolution by about two-thirds confirming hydrogen evolution by nitrogenase. Effect of carbon monoxide, noncompetitive inhibitor of hydrogenase and for acetylene reduction by nitrogenase,[2,7,16] was also examined. As can be seen, acetylene reduction was almost completely suppressed by CO but the rate of hydrogen evolution was not affected in compared with that of 90% Ar + 10% C_2H_2. From the result it is evident that hydrogen evolution is independent of hydrogenase. Similar cultures exposed to 10% O_2 and air showed the significant loss of both hydrogen evolving and acetylene reducing capacities confirmining

Table 1. Effect of Inhibiting Atmosphere on Hydrogen Evolution and Ethylene Production.[a]

Atmosphere	Rate of hydrogen evolution (ul/hr/mg cells (dry wt.))	%	Rate of ethylene production (ul/hr/mg cells (dry wt.))	%
100% Ar	51.7	(100.0)	—	—
99% Ar + 1% C_2H_2	45.5	(88.8)	8.1	(27.7)
90% Ar + 10% C_2H_2	35.1	(67.9)	29.2	(100.0)
40% Ar + 60% C_2H_2	16.5	(31.9)	30.4	(104.2)
100% C_2H_2	14.3	(27.7)	25.3	(86.6)
100% N_2	16.9	(32.7)	—	—
80% Ar + 10% CO + 10% C_2H_2	37.0	(71.6)	1.5	(5.1)
80% Ar + 10% O_2 + 10% C_2H_2	7.9	(15.3)	8.9	(30.5)
Air	2.9	(5.6)	—	—

[a] Incubation was stopped after 15 hrs.

Table 2. Hydrogen Evolution and Nitrogenase Activity in Resting Cells Prepared Differently.

Growth Media[b] \ Production Media[a]		Malate (30mM)			
		O	NH_4Cl (5mM)	CAM (20ug/ml)	Glutamate (7mM)
Malate	Glutamate	29.0[c](17.6)[d]	0(0)	23.2 (15.1)	55.7 (20.5)
	NH_4Cl	8.3 (7.4)	0(0)	2.2 (5.0)	30.9 (9.2)
	N_2 Gas	34.4 (13.9)	0(0)	5.2 (9.6)	97.1 (23.2)
	N_2 Gas and Glutamate	37.7 (18.0)	0(0)	8.8 (13.7)	73.2 (22.1)

a Incubation was stopped after 15 hrs.
b Each component was used at following concentrations:
DL-malate (30mM), L-glutamate (7mM), NH_4Cl (7mM), N_2 gas (30ml/min, bubbling).
c Rate of hydrogen evolution (ul/hr/mg cells (dry wt.))
d Rate of ethylene production (ul/hr/mg cells (dry wt.))

the relations described above.

Hydrogen evolution and nitrogenase activity in resting cells prepared from cultures of different nitrogen sources

Resting cells were prepared from cultures of different nitrogen sources and were assayed in four different reaction mixtures as shown in Table 2, to study the effect of nutritional history on hydrogen evolution and nitrogenase activity. When the reaction mixture contained only DL-malate (30mM), cells grown in NH_4Cl showed much decreased values of the rate of hydrogen evolution and nitrogenase activity than others. In addition, both of those were totally inhibited at 5mM of NH_4^+ ion added in the reaction mixtures. From the result it is evident that NH_4^+ ion acts as repressor for synthesis of nitrogenase as well as inhibitor of nitrogenase activity. If chloramphenicol was added before illumination was started, overall values of the rate of hydrogen evolution and nitrogenase activity decreased in compared with values in reaction mixtures of DL-malate. This might be due to the inhibition of protein synthesis and subsequent accumulation of small amount of NH_4^+ ion in cells through deamination of nitrogen compounds by chloramphenicol.[13] But, glutamate-grawn cells showed less inhibition by chorampenicol This indicates that glutamate may play an important role in the regulation of nitrogenase activity *in vivo* and further indicates that glutamine synthetase, a key enzyme in the metabolism of glutamate and NH_4^+ ion, may be involved in its regulation as proposed by Yoch[17] and Sweet and Burris.[18] The rate of hydrogen evolution and nitrogenase activity were largely enhanced when glutamate was added. This was due to the growth of resting cells and simultaneous synthesis of nitrogenase. Especially N_2-grown cells showed the highest values. Therefore, in order to increase the rate of hydrogen evolution and nitrogenase activity it can be one way to transfer cultures from N_2 to glutamate.

요 약

R. sphaeroides K-7에 의한 수소생성과 질소고정효소(nitrogenase)와의 연관성을 조사한 결과 수소생성은 질소고정효소에 의해 이루어지는 것으로 나타났다. 또한 수소생성은 수소효소(hydrogenase)와 무관하며 박테리오클로로필의 농도와도 무관한 것으로 나타났다. 글루타민산이 *in vivo*에서 질소고정효소의 활성도를 조절하는데 중요한 역할을 할수 있는 것으로 나타났으며 질소개스를 이용해 키운 세균을 글루타민산 존재하로 옮겼을때 보다 큰 정도의 수소생성능 및 질소고정효소 활성도가 나타났다.

Acknowledgements

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