Studies on Hydrogenases in Legume Root Nodule Bacteroids: Effect of ATP

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Hydrogenase activity (Tritium uptake activity) in cell-free extracts of bacteroids from soybean and lupin nodules was separated into two parts, whose activities were different from each other in responsiveness to ATP, by differential centrifugation, Sephadex gel filtration or DEAE-cellulose column chromatography. One was stimulated and the other was either inhibited or not affected by ATP.

Hydrogen evolution is found in most nitrogen-fixing organisms. The reaction is catalyzed by nitrogenase using ATP and reducing agents as well as by an usual hydrogenase.

In symbiotic nitrogen-fixing systems, only limited information is available with respect to hydrogenase system in comparison with hydrogen evolution by nitrogen-fixing system. Phelps and Wilson¹⁾ reported the presence of hydrogenase in pea root nodules. Dixon,^{2,3)} showed that legume root nodule bacteroids like the hydrogenase of *Azotobacter*, took up hydrogen and had no net hydrogen evolution. Hydrogenase in symbiotic nitrogen-fixing system is reported to play an important role in increasing the energy efficiency of nitrogen fixation.^{4,5)}

In previous studies,^{6~8)} we succeeded in obtaining cell-free extracts capable of uptaking hydrogen from legume nodules by manual grinding in liquid nitrogen, and described some properties of the hydrogenase preparations.

The present studies have shown that the hydrogenase activities in the cell-free extracts of bacteroids from soybean and lupin nodules are separated into two parts, whose hydrogenase activities were different from each other in responsiveness to ATP. One was stimulated and the other was either inhibited or not affected by ATP.

MATERIALS AND METHODS

Preparation of legume nodule extracts and the deter-

mination of hydrogenase activity generally followed the procedures previously described.

Legume root nodules. Soybean plants (Aosengoku and Norin No. 2) were grown in the field, and lupin plants (*Lupinus leuteus*, L.), in sand inoculated with *Rhizobium lupini* in a glasshouse kept at about 25°C. After 6 to 8 weeks, nodules of these plants were detached from the roots and put into liquid nitrogen to freeze immediately. These frozen nodules were kept in a bottle filled with nitrogen gas and stored in a freezer at -20°C.

The preserved nodules retained their hydrogenase activity for several months but their nitrogen-fixing activity was rapidly lost during the storage.

Preparation of extracts. The procedure is shown in Fig. 1. The nodules were ground in a chilled mortar under liquid nitrogen into powder. The powder was anaerobically suspended in a buffer solution containing 0.1 M phosphate, 10 mM MgCl₂, 20 mM ascorbate and 10% of the acid-washed polyvinylpyrrolidone (PVP), adjusted to pH 7.0 with KOH. The suspension was squeezed through four thicknesses of gauze, and the resulting filtrate was centrifuged at 5,500 g for 20 min. The precipitate, separated from the supernatant (S-1) and mainly composed of bacteroids, was suspended in 0.1 M phosphate buffer solution at pH 7.0 (P-1 fraction). The P-1 fraction was treated with a sonic oscillator at 20 KHz for 5 min under dropwise addition of liquid nitrogen, and the suspension of broken bacteroids was centrifuged at 15,000 g for 30 min at 2°C. The supernatant was designated as the S-2 fraction (cell-free extract), and the precipitate was suspended in 0.1 M phosphate buffer solution at pH 7.0, as the P-2 fraction. The S-2 fraction was further centrifuged at 184,000 g for 60 min at 2°C. The supernatant was designated as S-3 fraction, and the precipitate, the P-3 fraction, was suspended in 0.1 M phosphate buffer solution at pH 7.0.

FIG. 1. Fractionation of Legume Nodules.

Determination of hydrogenase activity. The extract of legume nodules was placed in the main chamber of a Warburg type vessel, and its final volume was adjusted to 5.0 ml with 0.1 M phosphate buffer at pH 7.0. 5 mg of sodium dithionite was added into the side arm of the vessel. The vessel was flushed three times with hydrogen gas and then filled with an appropriate gas mixture containing tritium gas. The reaction was begun by the addition of sodium dithionite into the main chamber, and the vessel was shaken at 30°C. After preincubation for 10 min, about 1.0 ml each of the reaction mixture was anaerobically removed from the vessel at intervals during the incubation. 0.5 ml of the aqueous mixture was placed in a liquid scintillation vial, and 10 ml of dioxane scintillator (10 g of PPO (2,5-diphenyloxazole), 0.25 g of POPOP [2,2'-p-phenylene-bis-(5-phenyl-oxazole)] and 100 g of naphthalene per liter of dioxane) and 0.1 ml of hyamine were added. The quantity of tritium taken up into the reaction mixture was calculated by subtracting the radioactivity at zero time from that at a designated time, which was one hour afterwards in most experiments. The tritium taken up in the reaction mixture linearly increased during incubation for at least 2 hr. Hydrogen uptake was calculated from the quantity of tritium in the reaction mixture solution. Hydrogenase activity is expressed in n moles of hydrogen taken up per hour. This method to detect the hydrogen uptake activity was

sufficiently reproducible. The amount of hydrogen taken up to the aqueous phase is in proportion to the amount of enzyme under the conditions used in these experiments.

Reaction gas mixture. H_2 , Ar, and CO used in these experiments were high quality commercial products, and the composition of reaction gas mixture was expressed in partial pressure in cmHg terms. 99.9% tritium gas was diluted with H_2 to 1 mC/ml and stored in gas container. The diluted tritium gas was added to the reaction vessels from 200 μ C to 500 μ C. The pressure in the vessel was adjusted to 1 atm with H_2 . In the experiment using CO, Ar was used as control.

ATP-generating system. The ATP-generating system contained, in final concentrations in the reaction mixture, 1.0 or 2.0 mM of adenosine triphosphate disodium salt (ATP), 3.0 or 6.0 mM of phosphocreatine disodium salt hexahydrate (PC), 20 or 40 μ g/ml of phosphocreatine kinase (PCK) and 2.0 or 4.0 mM of $MgCl_2$. All of them were made up in 0.1 M phosphate buffer and neutralized before use.

Protein determination. Protein was measured by the method of Lowry *et al.*,⁽⁹⁾ with bovine serum albumin used as standard.

DEAE-cellulose column chromatography. The following two methods were used in this experiment.

(1) A 1.8 by 12 cm column of DEAE-cellulose (Brown) with an exchange capacity of 0.91 meq/g was used. After this column was equilibrated with 0.025 M Tris-HCl buffer, pH 7.2, the S-2 (S)* fraction (containing 17.5 mg of protein) prepared from soybean nodule bacteroids with 0.025 M Tris-HCl buffer, pH 7.2, in place of 0.1 M phosphate buffer, pH 7.0, was applied from the top of the column, and the column was eluted successively with 30 ml of 0.025 M Tris-HCl buffer, pH 7.2, 50 ml each of the same buffer containing 0.15 M NaCl, then 0.25 M NaCl, and finally 80 ml of the same buffer containing 0.35 M NaCl. These solutions were previously degassed with a vacuum pump and bubbled with Ar. The eluate was collected in 12.5 ml fractions with a fraction collector, and the optical densities at 280 nm of these fractions were measured.

(2) The S-2 (L)** fraction prepared from lupin nodule bacteroids was passed through a Sephadex G-25 gel column (3 by 30 cm) equilibrated with 0.025 M phosphate buffer, pH 7.0, to adjust the phosphate concentration of the eluate to 0.025 M beforehand. The eluate (containing 74.2 mg of protein) was placed on a DEAE-cellulose column (1.8 by 25 cm) equilibrated

* (S) means that the preparation comes from soybean nodules.

** (L) means that the preparation comes from lupin nodules.

with 0.025 M phosphate buffer, pH 7.0. The column was eluted with 300 ml of a linear gradient of phosphate buffer (pH 7.0, from 0.025 M to 0.25 M). The eluate was collected in 15 ml fractions with a fraction collector, and the optical densities at 280 nm of these fractions were measured.

Some selected fractions eluted from these columns were immediately assayed for hydrogenase activity. To minimize the loss of activity during the operation, these fractionations were carried out as rapidly as possible (in about 2 hr).

Sephadex G-200 gel filtration. To the S-2 (L) fraction prepared from lupin nodules was added an equal volume of saturated ammonium sulfate solution adjusted to pH 7.0. The solution was centrifuged at 15,000 g for 20 min and the pellet was dissolved in a small amount of 0.025 M phosphate buffer (pH 7.0). Most of the hydrogenase activity in the S-2 (L) fraction was recovered in the 50% saturated ammonium sulfate pellet. The dissolved pellet containing 85 mg of protein in 5 ml was placed on a Sephadex G-200 gel column (3 by 26 cm) equilibrated with 0.025 M phosphate buffer (pH 7.0) at a flow rate of 20 ml per hour. The eluate was collected in 5.0 ml fractions with a fraction collector, and the optical density at 280 nm of these fractions were measured.

RESULTS

Distribution of hydrogenase activity in soybean nodules

The distribution of hydrogenase activity in the fraction prepared from soybean nodules in the flow diagram of Fig. 1 is shown in Table I. Most of the activity in the filtrate of

crushed nodule suspension was present in the P-1 (S) fraction (equivalent to the bacteriod fraction). After the P-1 (S) fraction was treated with a sonic oscillator, the hydrogenase activity was present only in the soluble S-2 (S) fraction. The S-2 (S) fraction was further fractionated by centrifugation at 184,000 g for 60 min at 2°C.

The hydrogenase activity in S-2 (S) fraction was distributed to S-3 (S) and P-3 (S) fractions at 54 and 43% respectively. In the three experiments described here, 87, 71, and 97% of the activity were recovered respectively. The losses of activity at the first two steps were considered to be caused by unstable property of the hydrogenase especially against oxygen.

Effect of ATP on the hydrogenase activity of the S-2 fraction

The cell-free nitrogen-fixing system required ATP for activity,¹⁰⁾ but the effect of ATP on the hydrogenase of nitrogen-fixing organisms remained to be investigated. Therefore the effect of ATP on the hydrogenase activity of the cell-free extracts of soybean and lupin nodules are examined. Since nitrogen-fixing system was reported to possess ATPase activity, an ATP generating system was used to keep the ATP concentration constant in the reaction mixture. Although hydrogenase activities of the S-2 fractions from both sources were apparently affected by ATP, the effect was variable for every preparation. In one case ATP was considered to inhibit the hydrogenase activity, but in another ATP seemed to stimulate the enzyme. When the effect of an ATP-generating system on hydrogenase of S-3 (S) and P-3 (S) fractions were examined, the activity of the S-3 (S) fraction was inhibited at 53% by the ATP-generating system, while that of the P-3 (S) fraction was slightly stimulated at 8% by the ATP-generating system. From this result, we assumed that the S-2 fraction contained two hydrogenase activities affected by an ATP-generating system. The following experiments confirmed this assumption.

TABLE I. DISTRIBUTION OF HYDROGENASE ACTIVITY IN VARIOUS FRACTIONS PREPARED FROM SOYBEAN NODULES

Fraction	Relative activity (%)	Relative protein (%) content	Specific activity (nmol/hr/mg protein)
Filtrate	100	100	1.9
S-1 (S)	7	67	0.2
P-1 (S)	80	32	4.4
P-1 (S)	100	100	2.3
S-2 (S)	71	52	2.3
P-2 (S)	0	57	0
S-2 (S)	100	100	4.2
S-3 (S)	54	83	2.8
P-3 (S)	43	24	7.5

TABLE II. INHIBITION OF HYDROGENASE ACTIVITY OF THE S-2 (S) FRACTION OF SOYBEAN NODULES BY CARBON MONOXIDE (CO)

Concentration of CO (cmHg)	Hydrogenase activity (nmol/hr/mg protein)		
	(—)	(ATP-Mg) ^a	(ATP-g.s.) ^b
0	11.0	8.2	7.7
5	7.4	9.3	12.0
10	0	7.9	—

^a ATP and MgCl₂ were added at 4 and 8 mM as final concentration respectively.

^b ATP-generating system. ATP, PC, PCK and MgCl₂ were contained at 1.0, 6.0 mM, 40 µg/ml and 2.0 mM respectively.

The concentration of protein in 5.0 ml of reaction mixture was 5.5 mg.

Inhibition of hydrogenase activity of the S-2 (S) fraction by carbon monoxide

Some hydrogenase activities from various microorganisms were reported to be inhibited by carbon monoxide (CO).^{11,12)} Therefore the influence of CO on the hydrogenase activity of S-2 (S) fraction was investigated. The relationship between the effects of CO and ATP on the hydrogenase activity was also investigated. The typical experimental results are shown in Table II. The hydrogenase activity of S-2 (S) fraction from soybean nodules was inhibited 30% by 5 cmHg CO and completely by 10 cmHg CO. However when ATP or ATP-generating system was added to the reaction mixture, no inhibition by CO was observed, or on the contrary, the hydrogenase activity assayed in the presence of CO showed a tendency to be activated by ATP or the ATP-generating system.

Sephadex G-200 gel filtration of the S-2 (L) fraction prepared from lupin nodules

Sephadex G-200 gel filtration of the S-2 (L) fraction of lupin nodules was carried out as described in METHODS. There were two peaks in the elution profile manifested by optical density at 280 nm (Fig. 2). The two peak fractions, SP-A (L), fraction collector tube number 4, 5, 6, (fraction 4~6) and SP-B (L) (fraction 20~22), were separately collected, and their hydrogenase activities were assayed both in the presence and absence of an ATP-

FIG. 2. The Elution Profile of Protein by the Sephadex G-200 Gel Filtration of S-2 (L) Fraction of Lupin Nodules.

TABLE III. HYDROGENASE ACTIVITY OF THE FRACTIONS FRACTIONATED FROM THE S-2 (L) FRACTION OF LUPIN NODULES BY SEPHADEX G-200 GEL FILTRATION

Fraction	Hydrogenase activity (nmol/hr/mg protein)	
	ATP-generating system ^a (—)	(+)
50% (NH ₄) ₂ SO ₄ pellet	0.31	0.37
SP-A (L)	0.49	0.58
SP-B (L)	6.40	3.30

^a ATP, PC, PCK and MgCl₂ were contained at 2.0, 3.0 mM, 20 µg/ml and 4.0 mM respectively in the reaction mixture.

The protein content in 5.0 ml of reaction mixtures of 50% (NH₄)₂SO₄ pellet, SP-A (L) and SP-B (L) were 8.5, 10 and 0.26 mg respectively.

generating system. The results are shown in Table III. The hydrogenase activity in SP-A (L) was slightly stimulated, but that of SP-B (L) was inhibited, by the ATP-generating system.

DEAE-cellulose column chromatography of the S-2 (S) fraction prepared from soybean nodules

The S-2 (S) fraction from soybean nodules was fractionated by DEAE-cellulose column chromatography as described in METHODS. There were four peaks in the elution profile manifested by the optical densities at 280 nm (Fig. 3), and the peak fractions in the elution pattern were designated as DP-A (S) (fraction

FIG. 3. The Elution Profile of Protein by the DEAE-Cellulose Column Chromatography of S-2 (S) fraction of Soybean Nodules.

TABLE IV. HYDROGENASE ACTIVITY OF THE FRACTIONS PREPARED FROM THE S-2 (S) FRACTION OF SOYBEAN NODULES BY DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

Fraction	Hydrogenase activity (nmol/hr/mg protein)	
	ATP-generating system ^a (—)	(+)
S-2 (S)	3.4	5.3
DP-A (S)	0	0
DP-B (S)	0	0
DP-C (S)	23.0	19.0
DP-D (S)	14.0	21.0

^a ATP, PC, PCK and MgCl₂ were contained at 2.0, 6.0 mM, 20 μg/ml and 2.0 mM respectively in the reaction mixture.

The protein content in 5.0 ml of reaction mixtures of S-2 (S), DP-A (S), DP-B (S), DP-C (S) and DP-D (S) were 4.48, 1.4, 1.78, 0.32 and 0.22 mg respectively.

3, 4), DP-B (S) (fraction 6), DP-C (S) (fraction 11) and DP-D (S) (fraction 15). Hydrogenase activities of these fractions were measured in the presence or absence of an ATP-generating system. The results are shown in Table IV. Hydrogenase activity was present only in the DP-C (S) and DP-D (S) fractions. The activity of the DP-C (S) was inhibited, but that of the DP-D (S) was activated, by the addition of the ATP-generating system.

DEAE-cellulose column chromatography of the S-2 (L) fraction prepared from lupin nodules

FIG. 4. The Elution Profile of Protein by the DEAE-Cellulose Column Chromatography of S-2 (L) Fraction of Lupin Nodules.

TABLE V. HYDROGENASE ACTIVITY OF THE FRACTION PREPARED FROM S-2 (L) FRACTION OF LUPIN NODULES BY DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

Fraction	Hydrogenase activity (nmol/hr/mg protein)	
	ATP-generating system ^a (—)	(+)
The eluate from Sephadex G-25 gel column	2.1	3.2
DP-A (L)	0	0
DP-B (L)	1.6	1.4
DP-C (L)	0	0.57

^a ATP, PC, PCK and MgCl₂ were contained at 1.0, 6.0 mM, 20 μg/ml and 2.0 mM respectively in the reaction mixture.

Protein contents in 5.0 ml of reaction mixtures of the eluate from Sephadex G-25 column, DP-A (L), DP-B (L) and DP-C (L) were 10.6, 6.1, 3.4 and 2.7 mg respectively.

DEAE-cellulose column Chromatography of the S-2 (L) fraction of lupin nodules was performed as described in METHODS. The elution profile manifested by the optical density at 280 nm, had three peaks (Fig. 4).

The peak fractions were designated as follows: fraction 4 as DP-A (L), fraction 8 as DP-B (L) and fraction 13 as DP-C (L). The hydrogenase activity was assayed in the presence or absence of an ATP-generating system. The results given in Table V show that hydrogenase activity existed only in DP-B (L) and DP-C (L) and the activity in the former was slightly inhibited, but that of the latter was stimulated, by the ATP-generating system.

DISCUSSION

Hydrogenase activities in the cell-free extracts of bacteroids from soybean and lupin nodules were measured by the amounts of tritium incorporated into the aqueous phase from the gas phase under anaerobic and reducing conditions. This tritium uptake method was worked out on the basis of the method to detect the fixation of molecular tritium which had been reported by Smith and Marshall.¹³⁾

In the present study, the effect of ATP on the hydrogenase activities in the cell-free extracts from soybean and lupin was investigated. According to the results described here, the hydrogenase activities in cell-free extracts prepared from both nodules seem to be similar to each other. Hydrogenases with different properties could be detected in these cell-free extracts. One activity was stimulated and another was either inhibited or unaffected by ATP. These results suggest that hydrogenase activities in cell-free extracts of both soybean and lupin nodule bacteroids consist of at least two isozymes or forms, which have different properties from each other. Probably, this is the cause for the irregularity of the effect of ATP on the hydrogenase activities in the cell-free extracts (S-2 fraction).

In the experiments on the effect of ATP on the hydrogenase activities, an ATP-generating system was usually used for keeping the ATP concentration in the reaction mixture during the incubation period. In the experiment on the inhibition of the hydrogenase activity by CO, the effects of ATP and an ATP-generating system on the hydrogenase activity seem to be similar to each other.

One of two hydrogenases in these cell-free extracts, interestingly, is stimulated by ATP. This hydrogenase has a larger molecular weight than the other which is inhibited by ATP as judged from ultra-centrifugation (Table I) and Sephadex G-200 gel filtration (Table III) studies. A previous investigation of the hydrogenase possessing the similar property only dealt with NAD reduction by the hydrogenase of a cell-free extract from *Hydrogenomonas*

eutropha.

In the case of *Hydrogenomonas eutropha*, it has been reported that ATP acts as a cofactor of NAD reduction by the hydrogenase.¹⁴⁾ But in the case of soybean and lupin nodules hydrogenases described in this report, we do not yet know how ATP affects the activity. This activity stimulated by ATP may be related to the hydrogenase reaction of the nitrogenase system which has been reported to be present in some nitrogen fixing microorganisms, for example, ATP-dependent hydrogen evolution,^{15, 16)} and ATP and nitrogen gas dependent hydrogen exchange reaction.^{17, 18)} However, the results of our experiments on the hydrogenase activity of *Azotobacter agile* indicated the tritium uptake activity to be stimulated by ATP was separated from the nitrogenase activity (acetylene reduction activity) by heat treatment and ultracentrifugation (in preparation). Therefore, the hydrogenase activity stimulated by ATP in these nodules also is considered to be catalyzed by hydrogenase. On this point, there is need for further investigation by using a more purified enzyme preparation.

The other hydrogenase which has a smaller molecular weight than the hydrogenase stimulated by ATP was either inhibited or unaffected by ATP, and this hydrogenase is assumed to be similar to hydrogenases in general which have been found in some microorganisms. The inhibiting effect of ATP on the hydrogenase activity may probably depend on the chelating effect of ATP.

The tritium uptake activity described here is supposed to indicated the hydrogen exchange reaction by hydrogenases because the activity was measured under anaerobic and reducing condition. But the possibility can not be excluded that this activity was expressed by hydrogen uptake into any hydrogen acceptors such as dithionite ($S_2O_4^{--}$) or sulfite (SO_3^{--}) come from sodium dithionite.

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