Role of Glutamine Synthetase as a Regulator of Nitrogenase in *Rhodopseudomonas sphaeroides* D-230

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광합성 세균에 있어서의 질소고정효소 합성 조절자로서의 Glutamine Synthetase의 역할

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Abstract: Optimum temperature and pH of glutamine synthetase activity (E.C. 3.6.1.2.) of *R. sphaeroides* D-230 was 35°C and 6.8, respectively. The adenylated state of GS in *R. sphaeroides* D-230 was stabilized by addition of 0.2 mg/ml of cethyltrimethylammoniumbromide. Valine, histidine, proline, isoleucine, and lysine were good nitrogen source for the growth of *R. sphaeroides* D-230. The growth of *R. sphaeroides* D-230 in N_2, $NaNO_3$ or NH_4Cl as sole nitrogen source was lower than in any other culture conditions. GS activity was inhibited, more or less, by various amino acid. The relative inhibition rate of the enzyme by added 7 mM arginine, NH_4Cl, N_2, and $NaNO_3$ was 63.8%, 26.79%, 6.24%, and 10.64%, respectively. The hydrogen evolution of *R. sphaeroides* D-230 was higher in N-limited media than in any other culture conditions. The hydrogen evolution of *R. sphaeroides* D-230 grown in N-limited media was inhibited by 0.1 mM MSX, irreversible GS inhibitor. GS activity was completely inhibited by 1.0 mM MSX but ammonia released maximally at the same concentration of MSX. Ammonia release by added MSX was increased up to 1.0 mM MSX, but decreased above 1.0 mM MSX. It is probably due to inhibition of nitrogenase activity by MSX. Nitrogenase activity was not inhibited at low concentration of MSX. These results suggests that the inhibition of nitrogenase activity by ammonia is mediated by products of ammonia assimilation rather than by ammonia itself.

Key Words: nitrogenase, glutamine Synthetase, *R. sphaeroides*

Nitrogenase activity of non-sulfur purple bacteria was regulated by short-term response upon addition of certain nitrogenous compounds. Ammonia or glutamine inhibits nitrogenase activity rapidly and completely (Jones and Monty, 1979; Sweet and Burris, 1981). The inhibition of nitrogenase by ammonia or glutamine is reversible and activity is fully restored on exhaustion of the nitrogen source (Arp and Zumft, 1983). Nitrogen fixation is controlled by ammonia-mediated process *in vivo* (Arp and Zumft, 1983; Sweet and Burris, 1981). Glutamine synthetase (GS) is a key enzyme in the assimilation of N_2 and ammonia by various organisms (Tyler, 1978). GS has been im-

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plicated in the genetic regulation of nitrogenase synthesis from studies with glutamine auxotroph (Brill, 1975; Streicher et al., 1974) and the use of GS inhibitor such as DL-methionine-DL-sulfoximine (MSX) (Arp and Zumft, 1983; Meyer and Vignais, 1979) and α-N-oxalyl-L-α,β-diaminopropionic acid (ODAP) (Meyer and Vignais, 1979). MSX rapidly abolished the ability of ammonia to inhibit the nitrogenase-mediated formation of hydrogen. The partial inhibition of hydrogen evolution by glutamine, however, remained unchanged (Arp and Zumft, 1983). This result suggests that the inhibition may be produced not by ammonia itself but by some products derived from the assimilation of ammonia. Streicher (1974) showed that glutamine auxotrophs are unable to synthesize nitrogenase during nitrogen limited growth. These results indicate a role of GS as a positive control element for nitrogen fixation. But detailed control mechanism of nitrogenase is unknown. Therefore, this report describes the roles of GS on the nitrogenase activity of *R. sphaeroides* D-230.

MATERIALS AND METHODS

Rhodopseudomonas sphaeroides D-230 was isolated from mud flats of paddy field in Kim Hae and Dae Jeo in Korea (Lee, 1986). The cell growth and culture conditions were described in the previous report (Lee 1986). Glutamate- or ammonia-grown cells were cultured in the media containing 7 mM glutamate or ammonium chloride as a nitrogen source. N-limited grown cells were cultured in nitrogen limiting media with various minerals (Lee, 1986) and 30 mM succinate. N_2-grown cells were cultured in nitrogen limiting media after bubbled with nitrogen gas instead of argon.

The activity of glutamine synthetase (E.C. 6.3.1.2) was measured by the r-glutamyltransferase assay method (Shapiro and Stadtman, 1970) except that 0.8M Tris-HCl (pH 7.0) was used instead of 1M imidazole-HCl buffer. The GS activity of *R. sphaeroides* D-230 in intact cells was measured by using 24 hour cultured cells. Cell-free extracts were prepared by destruction of the cells at 300 μA for 3 minutes in a ultrasonic generator (Nissei US300). The sonicated suspensions were centrifuged at 20,000 × g for 30 minutes. The resulting supernatant was used as an enzyme preparation of GS. Ammonia was measured colorimetrically by phenate method (APHA, 1981). Cell suspensions were centrifuged at 20,000 × g for 30 minutes at 4°C and 1 ml of the supernatant was used for the assay of ammonia.

Nitrogenase activity in whole cells was assayed by measuring hydrogen evolution at 30°C with Warburg mannometer (B. Braun V166). Hydrogen was measured under 100% argon gas which was scrubbed of oxygen by bubbling through a solution of 5% pyrogarol in 12 M NaOH. The center cell contained 0.2 ml of alkaline pyrogarols, 12.5 mg of pyrogarol in 0.2 ml of 10% KOH solution, and 15 discs (6.5 mm, diameter) of wattman filter papers. Main vessels contained 2 ml of cells. Illumination was provided by 100 W incandescent lamps at distance of ca. 15 cm below the vessel bottoms.

RESULTS AND DISCUSSIONS

Characterization of Glutamine Synthetase

The assimilation of ammonia by bacteria proceeds via either glutamate dehydrogenase or glutamine synthetase/glutamate synthase system, depending upon the organims and the ammonia concentration of the environment. And GS catalyzed

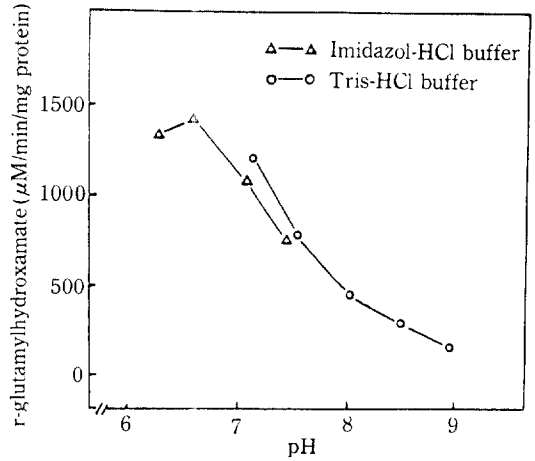

Fig. 1. *Optimum pH for glutamine synthetase activity in R. sphaeroides D-230.*

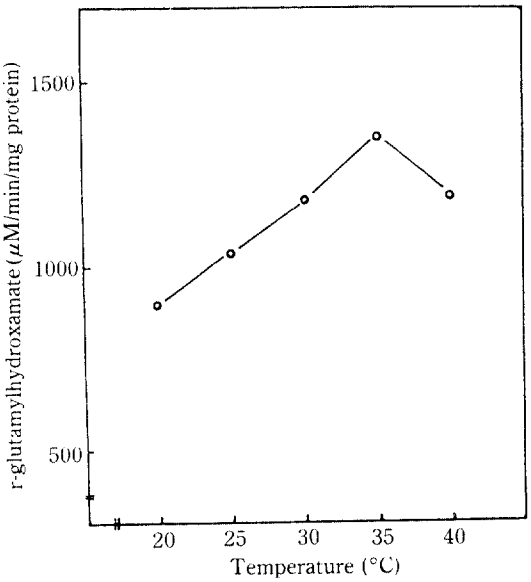

Fig. 2. *Optimum temperature for glutamine synthetase activity in R. sphaeroides D-230.*

the ATP-dependent production of glutamine from ammonia and glutamate (Tyler, 1978). The GS activity is regulated by the interconversion of active (deadenylated, GSI) and inactive (adenylated, GS II) forms depending upon the concentrations of various divalent cations (Tyler, 1978). The cumulative feedback inhibition of GS activity was reported in *R. capsulata* (Johansson and Gest, 1976). GS I was reversibly adenylated and its syntheis was repressed by added ammonium ions to culture medium (Johansson, 1977; Ludwig, 1980). The adenylation of GS decreased with increase of light intensity (Johansson, 1977). The effect of pH and temperature has been investigated in GS from *R. sphaeroides* D-230. The enzyme shows its highest activity at pH 6.8 (Fig. 1). The optimal temperature for the enzyme activity was 35°C (Fig. 2).

The adenylation state of GS can change markedly during harvesting of cells (Bender et al., 1977) and this can be prevented by pretreatment of cells with the detergent cethyltrimethylammoniumbromide (CTAB) (Johansson and Gest, 1977). Fig. 3 shows the effects of CTAB on stabilization of the adenylated state of GS. Before measuring of GS activity, various concentration of CTAB was added to the cells. The addition of 0.2 mg/ml of CTAB stabilized the adenylated state of glutamine synthetase of *R. sphaeroides* D-230.

Growth and GS activity with various nitrogen compounds

Table 1 shows the effect of nitrogen source on growth of *R. sphaeroides* D-230. Valine, histidine, proline, isoleucine, and lysine were good nitrogen sources for the growth of *R. sphaeroides* D-230. The growth of *R. sphaeroides* D-230 on N_2, $NaNO_3$ or NH_4Cl as a sole nitrogen source was lower comparing with those as shown in Table 1.

GS activity was the highest when *R. sphaeroides* D-230 was cultured in the absence of nitrogen sources. GS activity was inhibited, more or less, by various amino acid. The relative inhibition of the arginine (7 mM) on GS was 63.8%. When

Table 1. *Growth and GS activity of R. sphaeroides D-230 in media containing 30 mM succinate and 7 mM various nitrogen compounds.*

Nitrogen source	Growth*	GS activity	
		r-GH**	relative activity (%)
N-limited***	2.467	170.0	100
Alanine	2.547	144.3	81.98
Arginine	2.652	63.7	36.2
Glutamate	3.51	172.7	98.11
Glutamine	2.519	126.9	72.11
Glycine	2.48	123.7	70.29
Histidine	4.376	119.5	67.92
Isoleucine	4.093	127.1	72.24
Leucine	3.064	110.8	62.96
Lysine	4.016	124.5	70.74
Methionine	3.021	140.8	79.99
Phenylalanine	3.116	171.5	97.47
Proline	4.322	145.6	82.7
Serine	2.812	139.5	79.25
Valine	4.411	140.6	79.87
NH_4Cl	2.679	106.38	73.21
$NaNO_3$	2.261	157.3	89.36
N_2	2.49	165.0	93.76

*mg/ml
**γ-glutamylhydroxamate (μM/min/mg cell dry weight)
***The culture media did not contain nitrogen sources.

the cell was cultured in the media containg inorganic nitrogen source such as NH_4Cl, N_2, and $NaNO_3$, the relative inhibitory effect by these compounds on GS activity was 26.79%, 6.24% and 10.64%, respectively (Table 1). These results were similar to those of Johansson (1976) and Khanna (1983). Khanna and Nicholas (1983) showed that the purified GS of *Chlorobium vibrioforme f. thiosulfatophilum* was markedly inhibited by alanine, glycine, serine, and lysine, and these amino acids in various combinations showed cumulative inhibition. Johansson and Gest (1976) showed that the GS activity of *Rhodopseudomonas capsulata* was nortably inhibited by alanine, carbamylphosphate, glycine, histidine and tryptophan.

Inhibition of Glutamine Synthetase by MSX and Relationship to Nitrogenase Activity

Many heterotrophic anaerobes can utilize and produce H_2 through the action of hydrogenase (Gray and Gest, 1965). But in photosynthetic bacteria, the photoproduction of H_2 is catalyzed by nitrogenase, where as the utilization of H_2 as a biosynthetic reductant is effected by another kind of (classical) hydrogenase (Wall et al., 1975). Sweet and Burris (1981, 1982) reported the effects of NH_4^+ and DL-methionine- (DL) sulfoximine (MSX) on H_2 evolution by *Rhodospirillum rubrum* under five conditions of nitrogen availability. In

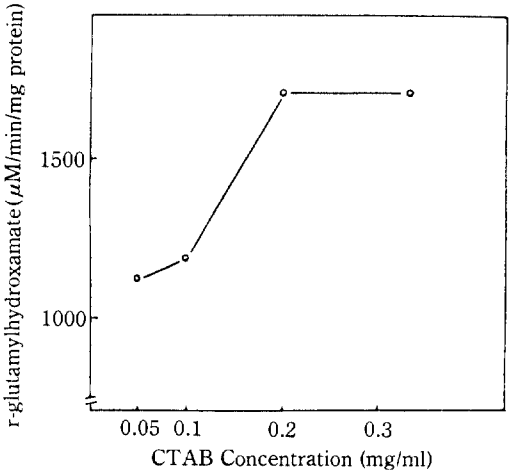

Fig. 3. *Stabilization of adenylation state of glutamine synthetse in R. sphaeroideds D-230 by pretreatment with cethyltrimethylammoniumbromide.*

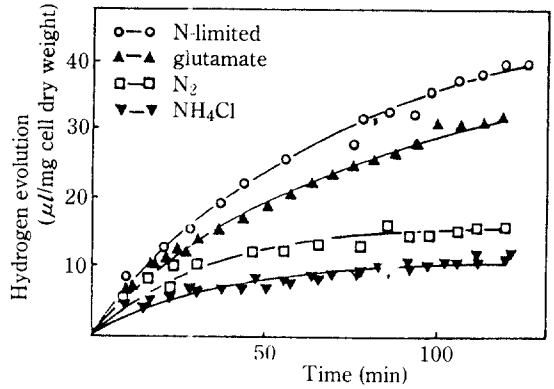

Fig. 4. *Hydrogen evolution of R. sphaeroides D-230 grown in four different media.*

cells grown on N_2, and NH_4Cl containing media, nitrogenase activity was relatively low. In contrast, cells grown in glutamate- and N-limited media showed higher nitrogenase activity. Table 1 showed cell growth and GS activity of *R. sphaeroides* D-230 grown under four different culture conditions, such as N_2, NH_4Cl, glutamate, N-limited media. The growth rate of *R. sphaeroides* D-230 was the highest in the glutamate-media among four culture conditions. The GS activities of *R. sphaeroides* D-230 grown under N-limited, glutamate-, N_2, and NH_4Cl-media were 176, 172.7, 165, and 106μM r-glutamylhydroxamate/min/mg cell dry weight, respectively. The lowest GS activity of *R. sphaeroides* D-230 grown on NH_4Cl-media may be due to the adenylation of GS in the presence of excess NH_4^+ (Johansson and Gest,

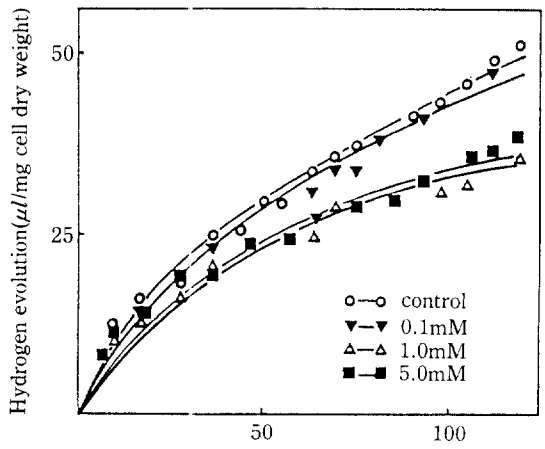

Fig. 5. *Effects of MSX on hydrogen evolution of R. sphaeroides D-230 grown in N-limited media.*

1977). The hydrogen evolution by *R. sphaeroides* D-230 grown under four different culture conditions was shown in Fig. 4. When *R. sphaeroides* D-230 was cultured in N-limited media, the hydrogen producing ability was higher than in any other culture conditions. The low hydrogen producing ability of N_2-grown cells may be due to N_2 gas. Nitrogen gas could be utilized as the sole nitrogen source for *R. sphaeroides* D-230. This suggested that photoproduction of hydrogen from *R. sphaeroides* D-230 was catalyzed by nitrogenase (Hillmer and Gest, 1977; Wall et al., 1975). Because NH_4^+ inhibited nitrogenase, nitrogenase activity of NH_4Cl-grown cells was low (Arp and Zumft, 1983; Streicher et al., 1974; Sweet and Burris, 1981).

MSX, irreversible GS inhibitor, is a structural analogue of glutamate (Flores et al., 1980). Fig. 5 shows the effect of MSX on hydrogen evolution of *R. sphaeroides* D-230 grown in N-limited media. The hydrogen evolution of *R. sphaeroides* D-230 grown in N-limited media was inhibited by 0.1 mM MSX. This result suggested that GS might be positive control element for nitrogen fixation in *R. sphaeroides* D-230. These result seems to be similar to previous reports (Meyer and Vignais, 1979; Streicher et al., 1974). Fig. 6 shows the effects of MSX on GS activity and ammonia release. GS was completely inhibited at 1.0 mM MSX, but ammonia release was maximum at the same concentration of MSX. Ammonia release by added MSX was increased up to 1.0 mM MSX, but decreased above 1.0 mM MSX. It is probably due to inhibition of nitrogenase activity by MSX. Nitrogenase activity was not inhibited at low concentration of MSX. These results suggest that the inhibition of nitrogenase activity by ammonia was mediated by products of ammonia assimilation rather than by ammonia itself. This result seems to be similar to previous reports (Arp and Zumft, 1983; Jones and Monty, 1979; Sweet and Burris, 1981).

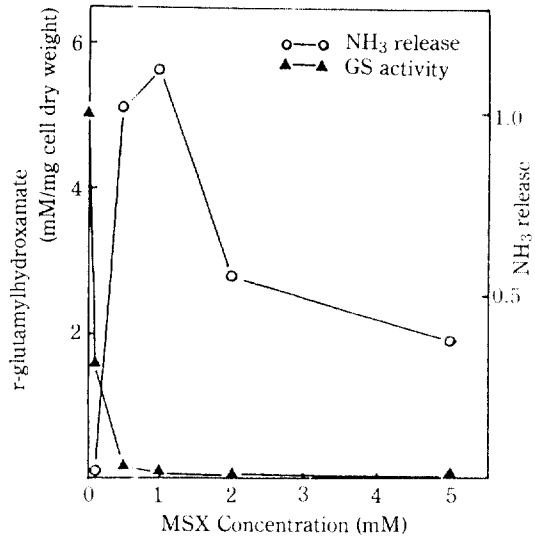

Fig. 6. *Glutamine synthetase activity and ammonia release in the presence of MSX.*

적 요

R. sphaeroides D-230의 glutamine synthetase 활성은 pH 6.8과 35℃의 온도에서 최대 활성을 나타냈으며, 추출과정에 0.2 mg/ml의 cethyltrimethylammoniumbromide를 첨가하였을 때 GS의 활성이 가장 안정하게 유지되었다. *R. sphaeroides* D-230을 최소배지에 30 mM succinate와 7 mM의 질소 화합물을 첨가하여 배양하였을 때, alanine, histidine, proline, isoleucine, lysine이 좋은 질소원으로 사용되었으며, N_2, $NaNO_3$, NH_4Cl을 첨가하였을 경우 성장이 저조하였다. GS의 활성은 대부분의 아미노산에 의해 다소 저해를 받았으며 특히 arginine의 경우 질소원을 첨가하지 않은 경우보다 63.8%의 저해를 받았다. 그리고 NH_4Cl, N_2, $NaNO_3$에 의해선 26.79%, 6.24%, 10.64%의 저해를 받았다. 수소생성은 질소원으로 N_2, glutamate, NH_4Cl을 첨가하였을 때보다 질소원을 첨가하지 않았을 경우 최대로 나타났다. GS의 저해제인 MSX를 1.0 mM첨가하였을 경우 수소생성은 감소하였고, GS의 활성은 완전히 저해되었으며, 그때 생성된 암모니아의 양은 최대로 나타났다.

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