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Janssen, Dick; Herst, Patricia M.; Joosten, Han M.L.J.; Drift, Chris van der

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Nitrogen Control in *Pseudomonas aeruginosa*: A Role for Glutamine in the Regulation of the Synthesis of NADP-Dependent Glutamate Dehydrogenase, Urease and Histidase

Dick B. Janssen, Patricia M. Herst, Han M. L. J. Joosten, and Chris van der Drift

Department of Microbiology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

Abstract. In *Pseudomonas aeruginosa* the formation of urease, histidase and some other enzymes involved in nitrogen assimilation is repressed by ammonia in the growth medium. The key metabolite in this process appears to be glutamine or a product derived from it, since ammonia and glutamate did not repress urease and histidase synthesis in a mutant lacking glutamine synthetase activity when growth was limited for glutamine. The synthesis of these enzymes was repressed in cells growing in the presence of excess glutamine. High levels of glutamine were also required for the derepression of NADP-dependent glutamate dehydrogenase formation in the glutamine synthetase-negative mutant.

Key words: Nitrogen control – Glutamate dehydrogenase – Urease – Histidase – Glutamine auxotrophs – Glutamine synthetase – *Pseudomonas aeruginosa*

In *Pseudomonas aeruginosa* several enzymes involved in the catabolism of organic nitrogen compounds are subject to catabolite repression by tricarboxylic acid cycle intermediates such as succinate or citrate. Under conditions of nitrogen limitation, however, the synthesis of some of these enzymes is derepressed, thus allowing the utilization of their substrates as nitrogen source. Histidase, urocanase (Potts and Clarke 1976) and agmatine hydrolase (Mercenier et al. 1980) are regulated in this way. Nitrogen limitation results also in derepression of glutamine synthetase (Janssen et al. 1980) and urease (Kaltwasser et al. 1972; Janssen et al. 1980), repression of NADP-dependent glutamate dehydrogenase (Brown et al. 1973; Janssen et al. 1980) and, in the presence of nitrate, in derepression of assimilatory nitrate reductase (Sias and Ingraham 1979).

The mechanism of this nitrogen control in *P. aeruginosa* is not known. In enteric bacteria a role for glutamine synthetase in nitrogen regulation was proposed by Magasanik and coworkers (Magasanik et al. 1974). In this model glutamine synthetase controls the synthesis of a number of enzymes involved in nitrogen assimilation e.g. histidase, proline oxidase, glutamate dehydrogenase, urease and glutamine synthetase itself. Glutamine and α -ketoglutarate were suggested to be key metabolites in this process by affecting the adenylylation state of glutamine synthetase and thereby its regulatory properties.

Offprint requests to: D. B. Janssen

The presence of another gene involved in the mechanism of nitrogen control was recently demonstrated in *Salmonella typhimurium* (Kustu et al. 1979) and *Escherichia coli* (Pahel and Tyler 1979). The regulatory gene was found to be closely linked to, but different from the structural gene for glutamine synthetase, and probably encodes a regulator protein that controls the transcription of genes for proteins subject to nitrogen control.

In this paper we describe the regulation of the synthesis of assimilatory NADP-dependent glutamate dehydrogenase, urease and histidase in mutants from *P. aeruginosa* lacking glutamine synthetase activity. It appears that, in contrast to the wild type strain, ammonia and glutamate no longer affect the formation of these enzymes in the glutamine synthetase-negative mutants. The results suggest a key role for glutamine or a metabolic product derived from it in the nitrogen control of this organism.

Materials and Methods

Bacterial Strains

Pseudomonas aeruginosa strain PAO 2175 was kindly supplied by Dr. H. Matsumoto and was used as the wild type strain (Matsumoto et al. 1978). It is a methionine-auxotrophic, catechol 1,2-oxygenase-negative derivative of strain PAO of Dr. B. Holloway. Strains PAO 4001 and PAO 4006 are glutamine-requiring derivatives of strain PAO 2175 and were obtained as described below.

Growth Media

Minimal salts medium was medium P as described by Leisinger et al. (1972) to which methionine was added at 15 mg/l. A carbon and a nitrogen source were added as indicated. pH after sterilization was 7.2. Glutamine solutions were prepared freshly and sterilized by filtration.

Isolation of Glutamine-Requiring Mutants

A nutrient broth culture was mutagenized by incubation with ethylmethanesulfonic acid (10 μ l/ml) for 1 h at 37°C without shaking. 1 ml of the mutagenized culture was added to 30 ml nutrient broth supplemented with glutamine and grown overnight at 37°C. The cells were harvested, washed, and starved for nitrogen for 3 h at 37°C in a medium containing citrate. Then, (NH₄)₂SO₄, glutamate and carbenicillin (2 mg/ml) were added and mutants were counterselected during 16 h at 37°C. The cells were washed and grown in a medium containing citrate, ammonia, glutamate and glutamine. After repeating the enrichment cycle, appropriate dilutions were spread on agar plates and mutants were identified by replica plating. Glutamine-requiring strains were purified and maintained on nutrient agar

supplemented with glutamine. Six independent glutamine auxotrophs were obtained.

Growth Conditions

For experiments in which enzyme levels were determined, growth media were inoculated with appropriately diluted precultures on nutrient broth containing glutamine. Cells were grown overnight at 37°C and harvested in the exponential phase of growth (optical density at 600 nm 0.3–0.5).

In some experiments cells were grown under glutamine limitation as described by Prival et al. (1973). Batch cultures of 400 ml were inoculated to an optical density at 600 nm of 0.03 with a washed suspension of cells pregrown on nutrient broth plus glutamine. From a sterile stock solution, glutamine was added with a peristaltic pump at a constant rate of 2 ml/h and the cells were grown at 37°C and harvested after 15 h. The glutamine concentration in the stock solution was adjusted to a value that gave limited growth to a final optical density at 600 nm of 0.3–0.5 after 15 h. Therefore, the stock solution contained 1 mg/ml glutamine when strain PAO 2175 was cultured and the medium contained no other nitrogen source. In experiments with strain PAO 4001 solutions of 1 mg/ml and 3 mg/ml were used for growth in media in the presence and absence of glutamate or casamino acids, respectively. Glutamine was added at a growth-limiting rate to cultures of strain PAO 4006 when the stock solution contained 15 mg/ml glutamine.

Methionine limitation was achieved in a similar way by employing a medium from which methionine was omitted.

Preparation of Cell Extracts

Cells were harvested by centrifugation, washed with 40 mM Na₂HPO₄ – KH₂PO₄ buffer (pH 7.0), resuspended in this buffer and disrupted by ultrasonic treatment. After centrifugation (45,000 × g for 30 min) a crude extract was obtained. When histidase levels were assayed the phosphate buffer was replaced by 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl (Lessie and Neidhardt 1967). Temperature was maintained at 0–4°C during all steps.

Enzyme Assays

γ-Glutamyltransferase activity of glutamine synthetase was measured at pH 7.9 (the isoactivity point of glutamine synthetase from strain PAO 2175) in cetyltrimethylammoniumbromide-treated cells as described before (Janssen et al. 1980). NADP-dependent glutamate dehydrogenase was measured by following the oxidation of NADPH spectrophotometrically (Meers et al. 1970) and urease by measuring ammonia production from urea. The incubation conditions have been described earlier (Janssen et al. 1980). Histidase was measured at 25°C by following the production of urocanic acid from histidine as described by Lessie and Neidhardt (1967). Protein was measured according to Lowry et al. (1951) with bovine serum albumin as the standard. One unit of enzyme is defined as the activity that forms 1 μmol of product per min under the incubation conditions used.

Materials

Amino acids were obtained from Merck, Darmstadt, FRG. Only L-amino acids were used. Vitamin-free casamino acids (acid hydrolysate) were from Difco Laboratories, Detroit, Mich. All other chemicals were of reagent or analytical grade.

Results

Isolation and Characterization of Glutamine-Requiring Mutants

Mutants from *Pseudomonas aeruginosa* PAO 2175 that require glutamine for growth were isolated as described under Materials and Methods. Two carbenicillin counter-selection cycles of 16 h were used to eliminate prototrophs and mutants with high reversion rates. Six independent glutamine auxotrophs were obtained (Gln⁻ phenotype). Growth of five of these mutants was found to be strictly dependent on the

presence of glutamine and no growth was observed on autoclaved nutrient broth unless glutamine was added. One strain showed leaky growth on media not containing glutamine and was not studied further. The five strict glutamine auxotrophs were found to lack glutamine synthetase activity, as measured by the transferase assay, when grown on a medium containing citrate and glutamine (see also Table 2). Two strains, designated PAO 4001 and PAO 4006, were chosen for further study.

Nitrogen Regulation in *P. aeruginosa* PAO 2175

The regulation of the synthesis of glutamine synthetase, NADP-dependent glutamate dehydrogenase and urease in *P. aeruginosa* was described before (Brown et al. 1973; Janssen et al. 1980). The data presented in Table 1 show that also in *P. aeruginosa* strain PAO 2175 glutamine synthetase and urease are repressed when the cells are grown on excess ammonia as compared with growth on a limiting nitrogen source such as a low concentration of nitrate. NADP-dependent glutamate dehydrogenase levels are elevated in the presence of ammonia and the enzyme is repressed when glutamate or glutamine are present in the growth medium. The data also show that glutamate and casamino acids are good nitrogen sources for *P. aeruginosa* and repress the formation of glutamine synthetase and urease.

In order to establish a possible relationship between glutamine synthetase activity and the regulation of glutamate dehydrogenase and urease, the synthesis of these enzymes has to be compared between the wild type strain and glutamine synthetase-negative mutants during growth under nitrogen-limited conditions. In *Klebsiella aerogenes* and *Escherichia coli*, nitrogen-limited growth could be obtained in batch cultures with 0.2% glutamine as sole nitrogen source (Prival et al. 1973; Pahel et al. 1978); however, by use of this concentration, or even a tenfold lower one, no derepression of glutamine synthetase was obtained in *P. aeruginosa* (Table 1). Also when the growth temperature was lowered to 30°C and the incubation time reduced to 8 h, no nitrogen limitation was observed with glutamine as sole nitrogen source. Apparently glutamine is a good nitrogen source and is assimilated much faster in *P. aeruginosa* than in enteric bacteria. Nitrogen limitation in the presence of glutamine could be achieved by slowly adding glutamine to batch cultures not containing other nitrogen sources and an excess of citrate as carbon source. In these cultures glutamine synthetase and urease became derepressed, while NADP-dependent glutamate dehydrogenase levels were low (Table 1).

Regulation of NADP-Dependent Glutamate Dehydrogenase and Urease in Glutamine Synthetase-Negative Mutants

When the Gln⁻ mutant PAO 4001 was grown under glutamine-limited conditions, urease was derepressed and NADP-dependent glutamate dehydrogenase repressed, just as observed for the wild type strain (Table 2). The addition of ammonia to the growth medium, which resulted in strong repression of urease in the wild type strain, did not cause repression of urease formation in mutant strain PAO 4001. Also the further addition of glutamate or casamino acids resulted neither in lowered urease levels nor in derepression of NADP-dependent glutamate dehydrogenase. The formation of urease was repressed by the addition of excess glutamine to the growth medium, which further caused an increase of the NADP-dependent glutamate dehydrogenase level. The obser-

Table 1. Regulation of glutamine synthetase, NADP-dependent glutamate dehydrogenase and urease in *Pseudomonas aeruginosa* PAO 2175 with different nitrogen sources

Growth medium ^a	Enzyme activities ^b		
	GS	GDH	urease
nit	330	11	1,890
amm	16	174	107
glu	22	18	60
cas	18	8	10
amm + glu	24	38	23
amm + gln	23	40	80
gln ₁	31	16	101
gln ₂	31	19	163
gln ₁ ^c	16	50	15
gln ₃	120	0	2,300

^a The growth medium contained 1% trisodiumcitrate. 2 H₂O as carbon source and a nitrogen source as indicated. nit = 0.02% KNO₃; amm = 0.2% (NH₄)₂SO₄; glu = 0.2% glutamic acid; cas = 0.5% casamino acids; gln₁ = 0.2% glutamine; gln₂ = 0.02% glutamine; gln₃ = glutamine added at a growth-limiting rate

^b GS (glutamine synthetase) activities are expressed in mU/mg dry weight; GDH (NADP-dependent glutamate dehydrogenase) and urease activities are given in mU/mg protein

^c In this experiment the growth temperature was reduced to 30°C and the cells were harvested after 8 h of growth

vation that glutamine, but not ammonia, glutamate or casamino acids, repress the synthesis of urease and derepress NADP-dependent glutamate dehydrogenase formation in a Gln⁻ mutant suggests that readily available nitrogen sources have to be converted to glutamine or some metabolic product derived from it in order to affect the synthesis of enzymes subject to nitrogen control. The high level of urease and the low level of NADP-dependent glutamate dehydrogenase are not the result of amino acid limitation in general, since methionine limitation of strain PAO 4001 in the presence of glutamine did not result in high urease levels or strong repression of NADP-dependent glutamate dehydrogenase (Table 2).

The regulation of urease and NADP-dependent glutamate dehydrogenase in the glutamine synthetase-negative strain PAO 4006 is different from that observed in strain PAO 4001 (Table 2). Urease levels were high under all growth conditions tested, even in the presence of excess glutamine in the medium. NADP-dependent glutamate dehydrogenase was severely repressed under all conditions.

In preliminary experiments performed with PAO 4006, it was observed that the glutamine requirement was much higher in this strain than in the other Gln⁻ mutants. When growth was measured in batch cultures containing citrate as carbon source and glutamine as sole nitrogen source, the wild type strain PAO 2175 needed 0.01% glutamine in the medium in order to reach a density of 0.2 mg cell dry weight/ml, while the Gln⁻ mutants PAO 4001 and PAO 4006 required 0.03% and 0.2% glutamine, respectively. Similar high levels of glutamine were required by strain PAO 4006 when glucose, succinate or glutamate replaced citrate as carbon source. This indicates that the requirement for high amounts of glutamine is not caused by the inability to assimilate citrate, which could be due to a defect in the tricarboxylic acid cycle. After growth of strain PAO 4006 on citrate medium supplemented with 0.2% glutamine, 90% of the nitrogen present in glutamine was recovered in the growth medium as ammonia, which indicates that glutamine is subject to rapid breakdown in this strain, due to a yet unknown defect.

Table 2. Regulation of NADP-dependent glutamate dehydrogenase and urease in mutants lacking glutamine synthetase activity

Strain	Growth medium ^a	Enzyme activities ^b		
		GS	GDH	urease
PAO 4001	gln ₁	0	0	4,400
PAO 4001	amm + gln ₁	0	0	3,140
PAO 4001	amm + glu + gln ₁	0	3	4,620
PAO 4001	amm + cas + gln ₁	0	1	3,980
PAO 4001	gln _c	0	15	108
PAO 4001	gln _c + met ₁	— ^c	22	6
PAO 4006	gln ₁	0	1	3,300
PAO 4006	amm + gln ₁	0	0	4,000
PAO 4006	gln _c	0	0	2,850
PAO 4006	amm + glu + gln _c	—	1	3,300
PAO 4006	amm + cas + gln _c	—	0	1,590

^a The growth medium contained 1% trisodiumcitrate. 2 H₂O as carbon source and a nitrogen source as indicated. amm = 0.2% (NH₄)₂SO₄; glu = 0.2% glutamic acid; cas = 0.5% casamino acids; gln₁ = glutamine added at a growth-limiting rate; gln_c = glutamine added in excess (0.2% for strain PAO 4001 and 1% for strain PAO 4006); met₁ = methionine added at a growth-limiting rate

^b GS (glutamine synthetase) activities are expressed in mU/mg dry weight; GDH (NADP-dependent glutamate dehydrogenase) and urease are given in mU/mg protein

^c —; not determined

Regulation of Histidase Formation

Histidase formation in *P. aeruginosa* is known to be regulated by induction, catabolite repression and derepression under nitrogen limitation (Lessie and Neidhardt 1969; Potts and Clarke 1976). This regulation pattern was confirmed in strain PAO 2175 (Table 3). The histidase activity was highest in cultures with histidine as sole carbon and nitrogen source. The enzyme was present at a repressed level in extracts from cells grown on histidine in the presence of citrate and ammonia. The repression of histidase was less when ammonia was omitted but increased when additional glutamine was included in the growth medium. Glutamine synthetase and urease levels were highest during growth in citrate plus histidine medium and were lowest in cells grown on medium containing citrate, ammonium and glutamine. Thus, under conditions of catabolite repression, histidase levels paralleled the glutamine synthetase and urease content of the cells. This suggests the presence of some common regulatory elements in the formation of these enzymes.

The mutants lacking glutamine synthetase activity showed abnormal regulation of histidase synthesis (Table 3). Strain PAO 4001 formed high levels of histidase and also urease when limited for glutamine in the presence of excess histidine. In contrast to the wild type strain, the addition of citrate and ammonia did not result in repression of the enzymes. Also glutamate and casamino acids could not repress histidase and urease. Similarly to urease formation, the synthesis of histidase was only repressed during growth with glutamine in excess. Thus, it appears that glutamine is also involved in the nitrogen control of histidase formation.

The Gln⁻ mutant PAO 4006 formed high histidase levels even in the presence of excess glutamine (Table 3).

Discussion

The results presented in this paper indicate the presence of a common regulatory mechanism in the nitrogen control of the

Table 3. Regulation of histidase in wild type PAO 2175 and the glutamine synthetase-negative mutants PAO 4001 and PAO 4006

Strain	Growth medium ^a	Enzyme activities ^b			
		GS	GDH	histidase	urease
PAO 2175		6	60	257	126
PAO 2175	cit	37	40	69	227
PAO 2175	cit + amm	12	62	46	141
PAO 2175	cit + amm + gln _c	10	24	32	61
PAO 4001	gln ₁	0	4	270	2,270
PAO 4001	cit + amm + gln ₁	0	3	203	1,920
PAO 4001	cit + amm + glu + gln ₁	— ^c	2	297	1,270
PAO 4001	cit + amm + gln _c	—	20	20	107
PAO 4006	cit + amm + gln _c	0	0	380	536

^a The growth medium contained 0.2% histidine with the additions indicated. cit = 1% trisodiumcitrate. 2 H₂O; amm = 0.2% (NH₄)₂SO₄; glu = 0.2% glutamic acid; cas = 0.5% casamino acids; gln₁ = glutamine added at a growth-limiting rate; gln_c = glutamine added in excess (0.2% for strains PAO 2175 and PAO 4001 and 1% for strain PAO 4006)

^b GS (glutamine synthetase) activities are expressed in mU/mg dry weight; GDH (NADP-dependent glutamate dehydrogenase), histidase and urease are given in mU/mg protein

^c —; not determined

synthesis of NADP-dependent glutamate dehydrogenase, urease and histidase in *Pseudomonas aeruginosa*. In the wild type strain, the cell contents of glutamine synthetase, urease and, under conditions of catabolite repression, also histidase parallel each other, while the level of NADP-dependent glutamate dehydrogenase is negatively correlated with these enzymes as shown before (Janssen et al. 1980; Brown et al. 1973; Potts and Clarke 1976). In mutants lacking glutamine synthetase activity, NADP-dependent glutamate dehydrogenase, histidase and urease were still correlated but the regulation differed from the wild type strain. In the Gln⁻ mutant PAO 4001 urease and histidase were no longer repressed and NADP-dependent glutamate dehydrogenase was not derepressed when cells were grown on excess ammonia, glutamate or casamino acids. Only the presence of excess glutamine in the medium led to a repression of urease and histidase and a derepression of NADP-dependent glutamate dehydrogenase. This suggests that glutamine plays a key role in the nitrogen control in *P. aeruginosa*.

The regulation pattern observed in strain PAO 4006 is distinct from that in strain PAO 4001 because the levels of urease, histidase and NADP-dependent glutamate dehydrogenase are not affected by an excess of glutamine. A rapid turnover of glutamine was observed and strain PAO 4006 required extraordinary high amounts of glutamine for growth. This implies that in PAO 4006 high levels of glutamine in the growth medium possibly do not correlate with high intracellular glutamine contents which could explain the lack of an effect of glutamine on the regulation of urease and NADP-dependent glutamate dehydrogenase. The phenotype of strain PAO 4001 should be characteristic for mutants in which only glutamine synthetase activity is lacking. Preliminary experiments with three of the other Gln⁻ mutants show that their properties are very similar to those of strain PAO 4001 (data not shown).

The regulation of NADP-dependent glutamate dehydrogenase requires some further comment. The enzyme is known to be formed at high levels in the presence of ammonia and repressed under nitrogen-limited conditions (Brown et al. 1973;

Janssen et al. 1980). The results presented here indicate that an elevated glutamine level, although possibly indirectly, may be the signal for the derepression of NADP-dependent glutamate dehydrogenase when ammonia is plentiful. On the other hand, the enzyme is repressed by glutamate or glutamine in the growth medium (Table 1), the latter presumably giving rise to an elevation of the intracellular glutamate pool. This suggests that the balance between the intracellular levels of glutamine and glutamate may be an important parameter in the regulation of NADP-dependent glutamate dehydrogenase. The repression mechanism affected by glutamate still seems to operate in the Gln⁻ mutant PAO 4001 since excess glutamine did derepress NADP-dependent glutamate dehydrogenase only to a level lower than that observed in the wild type strain during growth with excess ammonia as nitrogen source.

Our results do not exclude the possibility that a metabolic product derived from glutamine, rather than glutamine itself, is the effector that controls the formation of enzymes involved in nitrogen assimilation. The observation that casamino acids do not repress urease and histidase during glutamine limitation in a Gln⁻ mutant suggests that not one of the common amino acids (except glutamine, asparagine and tryptophan) is the responsible metabolite. It also should be noted that glutamine probably is not the sole effector of nitrogen control. The involvement of some aspect of carbon metabolism seems likely since carbon-limited growth, which presumably also results in lowered intracellular glutamine levels, does not promote derepression of urease and glutamine synthetase (Janssen et al. 1980).

A role for glutamine in the regulation of the enzymes involved in nitrogen assimilation has been shown for *Neurospora crassa*. Loss of glutamine synthetase activity impairs the repression of nitrate reductase (Premakumar et al. 1979) and uricase (Wang and Marzluf 1979) by ammonia and glutamate but not the repression by glutamine. Also in *Saccharomyces carlsbergensis*, ammonia itself is not the effector of nitrogen control (Van de Poll 1973). The presence of a positive control gene, *nit-2*, that regulates the synthesis of several enzymes involved in ammonia assimilation was demonstrated in *N. crassa* (Reinert and Marzluf 1975) but it is not known whether glutamine affects the expression of the *nit-2* gene product or has some other effect.

In enteric bacteria, glutamine synthetase was originally thought to be the regulator protein (Magasanik et al. 1974; Pahel et al. 1978) but the recent identification of a separate gene, closely linked to the structural gene for glutamine synthetase and required for the formation of transport proteins in *Salmonella typhimurium* (Kustu et al. 1979) and histidase in *Escherichia coli* (Pahel and Tyler 1979) indicates that another protein is involved. A role for glutamine in enteric bacteria is less evident although mutants of *Klebsiella aerogenes* that lack glutamine synthetase activity and constitutively form high levels of urease are known (Bender and Magasanik 1977; Friedrich and Magasanik 1977). In one of these mutants repression of urease and the inactive glutamine synthetase protein could be achieved in media containing glutamine and giving very strong nitrogen repression. Glutamine was proposed to be involved in signaling nitrogen repression of glutamine synthetase (Bender and Magasanik 1977).

In *Rhodospseudomonas capsulata* the loss of glutamine synthetase activity resulted in nitrogenase synthesis even in the presence of ammonia (Wall and Gest 1979), which indicates that also in this organism ammonia itself is not the corepressor in nitrogen control.

We suggest that in *P. aeruginosa* glutamine regulates the activity or synthesis of a protein that negatively or positively

affects the formation of enzymes subject to nitrogen control such as urease, histidase, NADP-dependent glutamate dehydrogenase and possibly also glutamine synthetase, nitrate reductase and allantoinase.

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