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Regulation of Nitrogen Fixation in *Azotobacter vinelandii* OP and in an Apparently Partially Constitutive Mutant

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Methylamine and 2-methylalanine appeared to act as co-repressors of nitrogenase in *Azotobacter vinelandii* OP. They inhibited the growth of this organism on molecular nitrogen but not on nitrate, ammonia, or Casamino Acids; they prevented the formation of nitrogenase by cells transferred from repression to induction conditions; and they did not inhibit the activity of nitrogenase in vitro. A mutant of strain OP, selected on the basis of its relative resistance to methylalanine, appeared partially constitutive because nitrogenase in this strain was less sensitive to repressors than was the enzyme in the wild-type strain.

Ammonia, peptone, and yeast extract have been shown to depress nitrogen fixation and its related hydrogen evolution in whole cells of *Azotobacter vinelandii* (8, 9, 13) and of *Rhodospirillum rubrum* (4–6, 17, 20). Ammonia has also been shown to delay the adaptation of *Aerobacter aerogenes* to growth on molecular nitrogen (18, 19). Ammonia and urea, singly or in combination, appear to repress nitrogenase in *Clostridium pasteurianum* (14, 15) and in *A. vinelandii* (10). The following activities related to nitrogen fixation also seem to be repressed by ammonia and urea: adenosine triphosphate (ATP)-dependent hydrogen evolution by extracts of *C. pasteurianum* (2, 11); ATP-dependent reduction of azide and hydrogen cyanide by extracts of *C. pasteurianum* and of *A. vinelandii* (10); and paramagnetic protein from nitrogen-fixing bacteria (12, 16).

It is not known whether ammonia, urea, nucleotides, and amino acids can all act as co-repressors or as feedback inhibitors or whether there are one or more mechanisms of repression of nitrogenase. Since the above compounds are interconvertible in whole cells, it is difficult to answer these questions.

Comparison between mutants constitutive for nitrogenase and the wild-type strain should help to elucidate the number of mechanisms of, and the number and type of components that are essential for, repression of nitrogenase. The use of nonmetabolizable co-repressors should obviate the question of interconversion of postulated co-repressors.

The first step in this investigation was to search for nonmetabolizable co-repressors of nitrogenase; the criteria in this search were (i) specific inhibition of growth on molecular nitrogen, (ii) no effect on nitrogenase activity in vitro, and (iii) inhibition of the formation of nitrogenase in cells transferred from repression to induction conditions. The molecules chosen as possible "gratuitous co-repressors" were all analogues of likely end products of nitrogen fixation. The next step was to isolate a nitrogenase-constitutive mutant; the criteria for such a mutant were (i) relative resistance to at least one of the gratuitous co-repressors, and (ii) relative resistance to repression by "nongratuitous" corepressors.

This communication reports the finding of a gratuitous repressor, as defined above, and of a partially nitrogenase-constitutive mutant.

MATERIALS AND METHODS

Bacterial strains. A. vinelandii strain OP (3) was kindly provided by P. W. Wilson and used as the wild-type strain. Strain 71-2 is a methylalanine-resistant mutant isolated in this laboratory from strain OP.

Culture conditions. Burk's nitrogen-free broth was the basic growth medium (21), with 2% glucose as carbon source, and with or without an additional source of nitrogen. For solid media, 1.5% agar (Difco) was added. Sterilization was with steam at 15 psi 121 to 132 C, for 15 min.

Liquid cultures, in Erlenmeyer flasks filled to one-fifth capacity, were incubated on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 27 ± 2 C with constant moderate agitation. Growth was followed turbidimetrically by use of a Klett-Summerson colorimeter with a no. 54 filter. One Klett unit is equivalent to 2.39×10^6 cells per ml.

A typical induction experiment was as follows. Cells

were pregrown into late logarithmic phase in shaking culture in a medium usually containing a co-repressor (growth medium), for 30 to 36 hr. They were then centrifuged and resuspended in twice the volume of nitrogen-free medium, with or without additions (induction medium), and were incubated as before for 12 to 13.5 hr. During the latter treatment, cells underwent between two and three divisions in an inhibitor-free medium and had again reached late log phase. The average net weight yields of cells from such experiments were 2.5 g/liter, 3 g/liter, and between 1.7 and 2.5 g/liter for organisms induced in nitrogen-free, Casamino Acids-containing, and inhibitor-containing induction media, respectively.

Extraction. Cells were harvested in a refrigerated centrifuge (Servall RC-2B) at 4,000 $\times$ *g* for 20 min and were subsequently ground in a mortar and pestle with Levigated Alumina (Spinco). The brei was suspended in 3 volumes of cold 0.025 M potassium phosphate buffer, *p*H 7.0, and subsequently packed at 3,000 $\times$ *g* for 20 min in a refrigerated instrument (Servall RC-2B). The resulting supernatant liquid was kept on ice until used as the crude extract within 4 hr after preparation. The protein concentration in these extracts was between 40 and 50 mg/ml.

Enzyme assays. Nitrogen fixation was measured by a slightly modified version of the procedure of Bulen, Burns, and Le Comte (1). The reactions were carried out in Warburg flasks in a Gilson respirometer at 30 C with moderate, constant shaking. The center well of each flask contained 0.20 ml of 20% KOH and a loose wad of cotton; the main vessel contained, in a final volume of 2.4 ml: 0.48 mg of creatine phosphokinase, salt-free grade (Sigma Chemical Co., St. Louis, Mo.), 125 μmoles of phosphocreatine pentahydrate, neutralized (Sigma Chemical Co.), 10 μmoles of Na_2 ATP, neutralized (Sigma Chemical Co.), 5 μmoles of $MgCl_2$, 50 μmoles of tris(hydroxymethyl)aminomethane (Tris)-chloride, *p*H 7.0, and 0.4 ml of crude extract. All flasks were flushed with argon (Canadian Liquid Air Ltd.) for 30 min with constant agitation. The stopcocks of the control flasks were then closed, and the apparatus was connected to a tank of nitrogen, purified grade (Canadian Liquid Air Ltd.). The test flasks were flushed for an additional 15 min with nitrogen, and subsequently the stopcocks were closed. A 0.2-ml amount of solution, containing 40 μmoles of $Na_2S_2O_4$, purified grade (Fisher Scientific Co., Fairlawn, N.J.), neutralized with NaOH, and which had been previously flushed for 30 min with nitrogen, purified grade (Canadian Liquid Air Ltd.), was added with a hypodermic needle to the side arm of each flask through a greased rubber stopper. After an equilibration period of 2 to 5 min, the reactions were started by tipping the dithionite into the main chamber of the Warburg flask. The rate of nitrogen fixation was constant during the 60-min incubation period. The reactions were stopped by letting air into the flasks. The contents were then introduced into Conway microdiffusion units (Falcon Plastics, Los Angeles, Calif.), and after addition of 1 ml of saturated K_2CO_3 the volatile ammonia was allowed to diffuse into 2% boric acid for 3 to 8 hr, at which time a sample of the ammonia-containing boric acid was reacted with commercially prepared Nessler's reagent (Paragon Co., Bronx, N.Y.), and the optical density at 440 mμ was compared to a standard.

One unit of activity of nitrogenase is defined as a difference of 1 mμmole of ammonia formed per minute between a reaction mixture incubated under a nitrogen atmosphere and its control incubated under an argon atmosphere. Activity was strictly proportional to extract concentration in the reaction mixture between 8 and at least 167 units. The specific activity of extracts from cells induced on N-free medium was between 1.8 and 2.4 mμmoles of net NH_3 formed per min per mg of protein.

Glucose-6-phosphate dehydrogenase was assayed by the procedure of Glock and McLean (7), with Tris-chloride instead of glycyl-glycine buffer. Activity was measured here as an enzyme and substrate-dependent increase in optical density at 340 mμ in a 1-cm light path length in a silica cuvette, as recorded by a Beckman DBG spectrophotometer. A unit of activity is defined as the reduction of 1 μmole of nicotinamide adenine dinucleotide phosphate per min. Measurements of activity were made in a range of protein concentration where the specific activity was constant and were recorded in the tables as units of activity contained in 1 ml of crude extract.

Selection of methylalanine-resistant mutants. Cells pregrown into the logarithmic phase were incubated without shaking, in the dark, at room temperature for 90 min, in the presence of 5 μg/ml final concentration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Inc., Milwaukee, Wis.) added in a small volume of ethyl alcohol. This resulted in 10% survival of cells. The bacteria were subsequently centrifuged, resuspended in 0.05 M potassium phosphate buffer, *p*H 7.0, and plated on solid nitrogen-free medium containing 2 mg of 2-methylalanine per ml (Eastman Chemical Products, Inc., Kingsport, Tenn.). Presumptive resistant mutants were then streaked on nonselective medium, and single clones were then isolated and tested for methylalanine resistance.

RESULTS AND DISCUSSION

Methylamine and methylalanine markedly inhibit the growth of *A. vinelandii* OP on molecular nitrogen but not on ammonia or Casamino Acids and not so much on nitrate (Table 1). If the above observation is due to the repression of nitrogenase by methylamine and by methylalanine, then growth in the presence of these inhibitors should be dependent on an externally supplied form of fixed nitrogen. This appears to be the case for ammonia (Fig. 1) and has been the case for nitrate and Casamino Acids as well. The control cultures in these experiments are not affected significantly by the concentration of fixed nitrogen in the culture; apparently, they are assimilating primarily atmospheric nitrogen. The cultures in which inhibitor is present do depend on the added fixed form of nitrogen, and hence, supposedly, are assimilating primarily fixed nitrogen.

Methylalanine and methylamine appear to in-

TABLE 1. *Effect of methylamine and methylalanine on growth on different N sources*

N source	Additions to medium[a]	Growth (percentage of control)[b]
N_2	None	100
	Methylalanine	5
	Methylamine	8
NH_4^+ (4 g/liter)	None	100
	Methylalanine	94
	Methylamine	86
NO_3^- (5 g/liter)	None	100
	Methylalanine	54
	Methylamine	63
Casamino Acids (5 g/liter)	None	100
	Methylalanine	105

[a] The amount of methylamine and methylalanine added was 1 g per liter.

[b] Growth was measure turbidimetrically. This is a composite of several experiments where 100% is between 170 and 330 Klett units.

hibit the formation of nitrogenase but not its in vitro activity (Tables 2–4). The extract mixtures were used in most of the experiments (Table 4) because methylamine and Casamino Acids interfere with the measurement of ammonia; consequently these "presumptive co-repressors" were not added directly to the assay mixture. Glucose-6-phosphate dehydrogenase was used as a control noninduced enzyme because it is not induced or repressed by the same conditions which induce or repress nitrogenase. It would appear from the foregoing that methylalanine and methylamine satisfy the three criteria for co-repressors outlined in the introduction.

Methylalanine-resistant mutants were derived from strain OP cells by treatment with *N*-methyl-

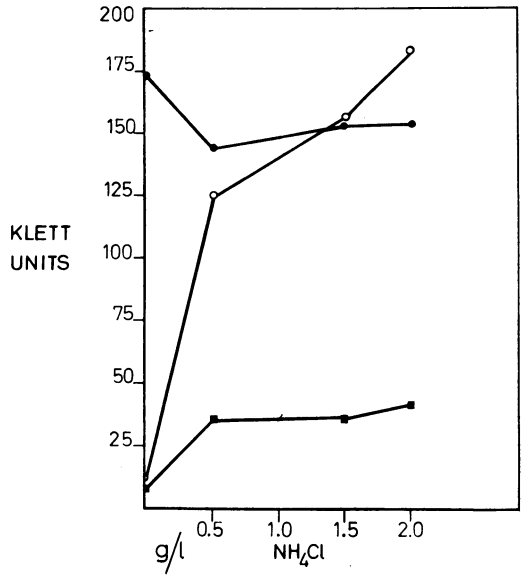

FIG. 1. *Effect of ammonia on growth of strain OP in the presence of no inhibitor (●), of 1 g of methylamine (○), and of 1 g of methylalanine per liter (■).*

TABLE 2. *Effect of methylalanine on induction of nitrogenase*[a]

Expt no.	N source in growth medium	Additions to induction medium	Enzyme activities: (a) Nitrogenase[b] (units/ml of extract)	Enzyme activities: (b) Glucose-6-phosphate dehydrogenase[b] (units/ml of extract)	Ratio of nitrogenase to glucose-6-phosphate dehydrogenase (a/b)
73	Yeast extract and Casamino Acids, 5 g/liter	None	39	6.7	5.8
		Methylalanine[c]	0.4[d]	11.4	—[d]
92	Casamino Acids, 5 g/liter	None	99	12.4	8.0
		Methylalanine[c]	35	8.5	4.1
96	NH_4Cl, 2 g/liter	None	79[e]	8.5	9.3
		Methylalanine[c]	13	7.2	1.8

[a] Cells were grown for 30 to 36 hr on N-free medium plus noted N sources (growth medium), centrifuged, and transferred to N-free medium plus noted additions (induction medium) for 12 to 13.5 hr.

[b] Both measurements were always made on the same extract.

[c] The amount of methylalanine added was 1g per liter.

[d] Equivalent to nil.

[e] The nitrogen source in the growth medium influences the maximum level of induction attained.

TABLE 3. *Effect of methylamine on induction of nitrogenase*

Expt no.	N source in growth medium	Additions to induction medium	Enzyme activities		Ratio of nitrogenase to glucose 6-phosphate-dehydrogenase (a/b)
			(a) Nitrogenase[a] (units/ml of extract)	(b) Glucose-6-phosphate dehydrogenase[a] (units/ml of extract)	
95	Casamino Acids, 5 g/liter	None	112	12.3	9.1
		Methylamine, 1 g/liter	67	12.1	5.5
103	NH_4Cl, 2 g/liter	None	68	6.6	10.3
		Methylamine, 1 g/liter	2	6.6	0.3

[a] As in Table 2.

TABLE 4. *Effect of presumptive co-repressors on nitrogenase activity in vitro*

Expt no.	Extract prepared from cells induced on	Additions to assay mixture	Units of nitrogenase activity (per ml of extract)
66 a	N-free medium	None	49
66 b	N-free medium	None	41
66 a	N-free medium	Methylalanine[a]	46
66 b	N-free medium	Methylalanine[a]	46
132	(i) Casamino Acids, 2.5 g/liter	None	10
	(ii) N-free medium	None	36
	1:1 mixture of i and ii	None	20
138	(i) N-free medium	None	43
	(ii) N-free medium + 1 g methylalanine per liter	None	8
	1:1 mixture of i and ii	None	27
140	(i) N-free medium	None	18
	(ii) N-free medium + 2 g methylamine per liter	None	1[b]
	1:1 mixture of i and ii	None	11

[a] The amount of methylalanine added was 1 mg/ml.
[b] Equivalent to nil.

N'-nitro-*N*-nitrosoguanidine as described in Materials and Methods and by subsequently plating the survivors on plates of nitrogen-free medium containing 2 g of methylalanine per liter. The resistant cells were then streaked under non-selective conditions, and single colonies were picked and tested for resistance to methylalanine. One of these, mutant no. 71-2, was relatively resistant to methylalanine and also partially resistant to methylamine (Table 5). The fact that methylalanine does inhibit the growth of this mutant shows that methylalanine can penetrate the 71-2 cells, but the data do not rule out the possibility that the mutant is partially impermeable to this compound. The relative resistance of this strain to methylamine suggested it might be constitutive if methylalanine and methylamine repressed nitrogenase by the same mechanism.

Casamino Acids digests appear to repress the nitrogenase of OP cells. They inhibit the formation of nitrogenase of this strain, but they do not

TABLE 5. *Effect of methylalanine and methylamine on growth of strain OP and mutant 71-2 in nitrogen-free medium*

Strain	Inhibitor	Concn	Growth (Klett units)[a]	
			Expt 1	Expt 2
		g/liter		
OP	None	—	320	198
	Methylalanine	1	12	7
	Methylalanine	2		7
	Methylamine	1	34	10
	Methylamine	2		4
71-2	None	—	236	170
	Methylalanine	1	216	137
	Methylalanine	2		90
	Methylamine	1	54	64
	Methylamine	2		5

[a] One Klett unit is equivalent to 2.39×10^6 cells per ml.

TABLE 6. *Effect of natural repressors on induction of nitrogenase in wild-type and a methylalanine-resistant mutant*

Expt no.	Strain	N source in growth medium	Additions to induction medium	Enzyme activities		Ratio of nitrogenase to glucose-6-phosphate dehydrogenase (a/b)
				(a) Nitrogenase[a] (units/ml of extract)	(b) Glucose-6-phosphate dehydrogenase[a] (units/ml of extract)	
67	OP	Casamino Acids, 5 g/liter	Not induced[b]	3, 2	8.2	0.2
		N_2	Not induced[b]	65, 68	8.2	8.0
121	OP	Casamino Acids, 5 g/liter	Casamino Acids, 2.5 g/liter	23	7.2	3.2
			None	117	9.7	12.1
112	71-2	Casamino Acids, 5 g/liter	Not induced[b]	32	19.3	1.7
		N_2	Not induced[b]	120	14.5	8.3
122	71-2	Casamino Acids, 5 g/liter	Casamino Acids, 2.5 g/liter	42	8.4	5.0
			None	43	6.7	6.4
126	71-2	Casamino Acids, 5 g/liter	Casamino Acids, 2.5 g/liter	46	7.7	6.0
	OP	Casamino Acids, 5 g/liter	Casamino Acids, 2.5 g/liter	4	8.7	0.5

[a] As in Table 2.
[b] Cells were harvested from growth medium, extracted and assayed directly.

inhibit its activity in vitro (Table 6, experiments 67, 121, 126; and Table 4, experiment 132). The nitrogen-fixing apparatus of mutant 71-2 seems less sensitive to repression by Casamino Acids than that of wild type (Table 6); consequently, it is believed to be partially nitrogenase-constitutive. The fact that the nitrogen-fixing apparatus of strain 71-2 is partially repressed by high levels of Casamino Acids (Table 6, experiment 112) indicates that the mutant is permeable to at least some of the components in the mixture.

The data reported in this paper indicate that methylalanine, methylamine, and Casamino Acids can all act as co-repressors of nitrogenase and that mutant 71-2 is partially nitrogenase constitutive.

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