Regulation of Enzyme Synthesis in the Arginine Biosynthetic Pathway of *Pseudomonas aeruginosa*

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In *Pseudomonas aeruginosa* the synthesis of only two out of eight arginine biosynthetic enzymes tested was regulated. Comparisons were made between the specific activities of these enzymes in bacteria grown on arginine or on its precursor, glutamate. *N*²-Acetylornithine 5-aminotransferase (ACOAT), an enzyme involved in both the biosynthesis and catabolism of arginine, was induced about 14-fold during growth of the organism on arginine as the only carbon and nitrogen source, and the anabolic ornithine carbamoyltransferase (aOTC), a strictly biosynthetic enzyme, was repressed 18-fold. Addition of various carbon sources to the arginine medium led to repression of ACOAT and to derepression of aOTC. Fructose, which supported only slow growth of *P. aeruginosa*, had a weak regulatory effect on the synthesis of the two arginine enzymes while citrate, a good carbon source for this organism, had a strong effect. The repression of ACOAT by citrate was not relieved by adding cyclic AMP to the medium. Under a variety of growth conditions leading to different enzyme activities, a linear relationship between the reciprocal of the specific activity of ACOAT and the specific activity of aOTC was observed. This inverse regulation of the formation of the two enzymes suggested that a single regulatory system governs their synthesis. Such a view was supported by the isolation of citrate-resistant regulatory mutants which constitutively formed ACOAT at the induced level and aOTC at the repressed level.

INTRODUCTION

Studies on the regulation of amino acid metabolism, conducted with *Pseudomonas aeruginosa* and *P. putida*, have revealed that these organisms have complex feedback mechanisms regulating the activity of allosteric enzymes in amino acid biosynthetic pathways. Regulation of enzyme synthesis, however, seems to be rare in amino acid biosynthesis whereas it is well documented for several pathways of amino acid catabolism (Clarke & Ornston, 1975). These generalizations apply to the regulation of arginine metabolism in *Pseudomonas* (Fig. 1). Inhibition of the first two enzymes of arginine biosynthesis by arginine, by arginine analogues and by several other effectors has been observed with purified enzyme preparations from *P. aeruginosa* (Haas *et al.*, 1972; Leisinger *et al.*, 1974; Haas & Leisinger, 1975). In the same organism Isaac & Holloway (1972) found up to 100-fold repression of the anabolic ornithine carbamoyltransferase (enzyme 6, Fig. 1) by arginine, whereas the activities of three other arginine biosynthetic enzymes (enzymes 2, 5' and 8, Fig. 1) were not affected by arginine limitation or arginine excess. Voellmy & Leisinger (1975) reported 15-fold induction of acetylornithine 5-aminotransferase (enzyme 4, Fig. 1) by arginine. In *P. putida*, *N*-acetylglutamate 5-phosphotransferase and/or *N*-acetylglutamate-5-semialdehyde dehydrogenase (enzymes 2 and 3, Fig. 1) as well as the anabolic ornithine carbamoyltransferase were slightly derepressed during arginine starvation (Condon *et al.*, 1976) and *N*²-acetylornithine 5-aminotransferase was induced by arginine (Chou & Gunsalus,

Fig. 1. Arginine metabolism in *P. aeruginosa*. Numbers 1 to 8 designate the arginine biosynthetic enzymes whose trivial and systematic names are listed in Table 1. The dashed line indicates arginine catabolism by the 'deiminase-pathway' involving enzymes: 9, arginine deiminase [L-arginine iminohydrolase; EC 3.5.3.6]; 10, catabolic ornithine carbamoyltransferase [carbamoylphosphate:L-ornithine carbamoyltransferase; EC 2.1.3.3]; 4, [EC 2.6.1.11; see Table 1]; 11, L-pyrroline dehydrogenase [L-pyrroline-5-carboxylate:NAD⁺ oxidoreductase; EC 1.5.1.12].

1971). In contrast to this limited control of enzyme synthesis in arginine biosynthesis, the enzymes of three different degradative pathways for arginine have all been reported to be inducible by arginine (Clarke & Ornston, 1975).

In the present study we have confirmed that in *P. aeruginosa* the synthesis of only two out of eight arginine biosynthetic enzymes tested, *N*²-acetylornithine 5-aminotransferase (ACOAT) and the anabolic ornithine carbamoyltransferase (aOTC), is regulated to a marked extent by arginine. The expression of these two enzymes is regulated in a strictly inverse manner by arginine and by different carbon sources, suggesting that a common regulatory system governs their synthesis. Some of the results presented in this paper have been reported in preliminary communications (Voellmy & Leisinger, 1972, 1976a).

METHODS

Bacterial strains. Experiments were done with the wild-type strain *Pseudomonas aeruginosa* PAO1 (ATCC 15692), the proline-auxotrophic strain PAO951 (*pro-137*), isolated after mutagenesis of strain PAO1 with 1-methyl-3-nitro-1-nitrosoguanidine, and the citrate-resistant mutant PAO959 (*pro-137*, CIT-r), which was derived from strain PAO951 by mutagenesis with ethyl methanesulphonate. (The mutation *pro-137* causes a defect in the proline-inhibitable ATP:L-glutamate 5-phosphotransferase, the first enzyme of proline biosynthesis; R. V. Krishna, unpublished work.)

Media. Medium P, a nitrogen-free minimal salts medium, was described by Leisinger *et al.* (1972). Carbon and nitrogen sources were added after sterilization from neutralized stock solutions in various combinations as indicated in Results. Glucose and fructose were used at 28 mM, L-amino acids and citrate at 20 mM and $(\text{NH}_4)_2\text{SO}_4$ at 15 mM. Solid media were prepared by inclusion of 1.6 % (w/v) Difco agar.

Growth conditions. Cultures were incubated at 37 °C. Bacteria for enzyme assays were grown in 2 l Fernbach flasks containing 1 l medium on a rotary shaker (170 rev. min⁻¹) with an eccentricity of 3 cm. They were harvested in the late-exponential phase, washed with saline [0.85 % (w/v) NaCl] and either used immediately or stored at -20 °C. Batches of strains PAO951 and PAO959 were routinely tested for the presence of prototrophic revertants by plating samples (approx. 5×10^8 cells) on minimal medium plates. Growth rates were determined with 5 ml cultures in test tubes as described by Leisinger *et al.* (1974).

Mutant isolation. The isolation of citrate-resistant (CIT-r) mutants was based on the fact that citrate represses ACOAT, which also catalyses the conversion of ornithine to glutamate 5-semialdehyde (Voellmy & Leisinger, 1975). Therefore a mutant with an early block in proline synthesis (e.g. PAO951) is unable to derive glutamate 5-semialdehyde from ornithine in the presence of citrate (see Fig. 1) whereas CIT-r derivatives have gained this ability. Spontaneous CIT-r mutants were selected by plating 0.1 ml of a washed suspension (2×10^8 cells ml⁻¹) of strain PAO951 on plates of medium P plus 20 mM-ornithine plus 20 mM-citrate. Alternatively, we treated strain PAO951 with ethyl methanesulphonate as described by Watson & Holloway (1976) before plating on the selective medium. After incubation for 2 d, large colonies were selected and tested for proline-auxotrophy. Prototrophs (approx. 50 %) were discarded. In the remaining isolates the activity of ACOAT during growth on medium P plus 20 mM-ornithine plus 20 mM-citrate was tested. In about 50 % we detected increased activities of ACOAT. The remaining 50 % showed wild-type ACOAT activities on the ornithine/citrate medium and were discarded.

Preparation of extracts and enzyme assays. The buffers used in the preparation of extracts for the various enzyme assays have been described by Haas *et al.* (1977). In the experiments reported in this paper we used 0.1 M-Tris/HCl pH 7.0 containing 0.1 mM-pyridoxal phosphate for the preparation of extracts which were to be assayed for aOTC and ACOAT. Extracts were prepared by suspending wet cells in buffer at 20 % (w/v) and subjecting them to ultrasonic disruption in an MSE 150 W ultrasonic disintegrator (eight bursts of 15 s each). After removal of cell debris by centrifugation at 30000 g for 20 min, the crude extracts were either dialysed overnight against the extraction buffer or desalted by passage through Sephadex G-25.

The methods used for assaying the enzymes of arginine biosynthesis have been listed by Haas *et al.* (1977). One unit (U) of enzyme is defined as that quantity of enzyme which catalyses the formation of 1 μmol of product per h under standard assay conditions. All enzyme activities reported represent the mean from at least two determinations in extracts from independently grown cells. For some experiments an ACOAT assay with a 10-fold increased sensitivity had to be used. It was based on the standard colorimetric assay described by Voellmy & Leisinger (1975). The incubation mixture (total volume 1.25 ml) contained: 1.0 ml extract prepared in 0.02 M-potassium phosphate buffer pH 7.0; 130 mM-Tris/HCl pH 9.0 at 25 °C; 19 mM-*N*²-acetyl-ornithine; 19 mM-2-oxoglutarate; 0.1 mM-pyridoxal phosphate. The pH of the reaction mixture containing all components except the enzyme was adjusted to 8.5 at 25 °C. The reaction was started by adding the prewarmed mixture of reagents to the enzyme; after 30 min incubation at 37 °C it was stopped by adding 0.5 ml 10.3 M-HCl. The solution was then boiled for 45 min, cooled to room temperature, and solid sodium acetate (1 g) was added to each tube, bringing the total volume of the mixture to 2.3 ml. Colour was developed by adding 0.2 ml 33 mM-*o*-aminobenzaldehyde and 0.35 ml distilled water.

Amidase activity was determined by the transferase assay according to Brammar & Clarke (1964).

Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Protein contents of growing bacteria were determined by measuring A_{546} and using an appropriate standard curve.

The sources of the chemicals used were as described by Voellmy & Leisinger (1975).

Table 1. *Regulation of synthesis of arginine biosynthetic pathway enzymes in P. aeruginosa* PAO1

No. in Fig. 1	Enzyme Trivial and systematic name	Specific enzyme activity [U (mg protein) ⁻¹] after growth on:	
		Medium P + glutamate*	Medium P + arginine*
1	<i>N</i> -Acetylglutamate synthase [acetyl-CoA: L-glutamate <i>N</i> -acetyltransferase; EC 2.3.1.1]	0.28†	0.28†
2	<i>N</i> -Acetylglutamate 5-phosphotransferase [ATP: <i>N</i> -acetyl-L-glutamate 5-phosphotransferase; EC 2.7.2.8]	1.92‡	1.86‡
3	<i>N</i> -Acetylglutamate-5-semialdehyde dehydrogenase [<i>N</i> -acetyl-L-glutamate-5-semialdehyde:NADP ⁺ oxidoreductase (phosphorylating); EC 1.2.1.38]	1.40	1.15
4	<i>N</i> ² -Acetylornithine 5-aminotransferase (ACOAT) [<i>N</i> ² -acetyl-L-ornithine:2-oxoglutarate aminotransferase; EC 2.6.1.11]	4.8	73.00
5	Acetylornithinase [<i>N</i> ² -acetyl-L-ornithine amidohydrolase; EC 3.5.1.16]	0.58	0.53
5'	Ornithine acetyltransferase [<i>N</i> ² -acetyl-L- ornithine:L-glutamate <i>N</i> -acetyltransferase; EC 2.3.1.35]	0.44	0.48
6	Anabolic ornithine carbamoyltransferase (aOTC) [carbamoylphosphate:L-ornithine carbamoyltransferase; EC 2.1.3.3]	4.79	0.27
7	Argininosuccinate synthetase [L-citrulline: L-aspartate ligase (AMP-forming); EC 6.3.4.5]	ND	ND
8	Argininosuccinase [L-argininosuccinate arginine-lyase; EC 4.3.2.1]	0.22	0.15

ND, Not determined. * At 20 mM. † Values from Haas *et al.* (1972). ‡ Values from Haas & Leisinger (1975).

RESULTS

The synthesis of two arginine biosynthetic enzymes is regulated by arginine

When the wild-type strain PAO1 was grown on medium P with either arginine or glutamate as the only carbon and nitrogen source, we observed about 14-fold induction of ACOAT and about 18-fold repression of aOTC by arginine (Table 1). The activities of enzymes 3 and 8 (see Fig. 1) were repressed very slightly, but reproducibly, by arginine whereas the activities of enzymes 1, 2, 5 and 5' were not altered (Table 1). As a control, a double auxotrophic strain with a requirement for leucine and lesions in enzymes 2 and 7 of the arginine pathway was grown in a chemostat (growth rate 0.2 h⁻¹) on minimal medium E (Vogel & Bonner, 1956) either under leucine limitation with an excess of arginine (repressive conditions) or under arginine limitation with an excess of leucine (derepressive conditions). Among seven arginine enzymes tested, the activities of only ACOAT and aOTC were altered by a factor of greater than two in response to changes in the arginine supply. It thus seemed unlikely that the lack of derepression of most of the arginine biosynthetic enzymes on medium P with glutamate was due to a high concentration of endogenously formed arginine.

The induction of ACOAT by arginine has been explained earlier by the dual role of this enzyme in arginine biosynthesis and in the catabolism of arginine via ornithine and glutamate 5-semialdehyde (Voellmy & Leisinger, 1975, 1976*b*). In order to exclude the possibility that there are two ACOATs, one biosynthetic and one degradative, we compared the chromatographic properties, the sedimentation behaviour in sucrose gradients, the electrophoretic mobility, the substrate specificity, and the inhibition pattern by substrate analogues

Fig. 2. Induction of ACOAT and repression of aOTC by arginine. Strain PAO1 was grown in a Fernbach flask on medium P with 20 mM-glutamate. At intervals, 5 ml samples were removed for the determination of ACOAT, aOTC and protein. ACOAT was determined by the assay procedure with a 10-fold increased sensitivity (see Methods). The arrow indicates the addition of arginine to the growing culture up to a final concentration of 20 mM. ●, ACOAT; ○, aOTC.

of ACOAT from arginine-grown cells with ACOAT from glutamate-grown cells. There were no detectable differences between the enzymes from the two sources (R. Voellmy and Th. Leisinger, unpublished work). We thus conclude that *P. aeruginosa*, in contrast to *Klebsiella aerogenes* (Friedrich *et al.*, 1978), forms only one ACOAT. Induction of ACOAT by arginine was not restricted to *P. aeruginosa* PAO; we have also observed a 15-fold induction of the enzyme by arginine in *P. aeruginosa* strains PAC and PAT.

The differential rates of synthesis of both ACOAT and aOTC changed immediately on addition of arginine to a culture growing on medium P with 20 mM-glutamate (Fig. 2). When chloramphenicol at 500 µg ml⁻¹ was added 1 min prior to arginine, the activities of ACOAT and aOTC remained unchanged for at least 3 h. These observations indicated that the two enzymes were metabolically stable and that the changes observed in their activities on addition of arginine were indeed due to induction and repression respectively.

Regulation of enzyme synthesis by carbon sources is superimposed on arginine-specific regulation

The specific activity of ACOAT was lower in cultures grown in a medium containing arginine plus an additional carbon source than in a culture grown on arginine alone (Table 2). (NH₄)₂SO₄ was included in all media in this series of experiments to eliminate possible regulatory effects caused by the availability of nitrogen. The severity of the repression of ACOAT by carbon compounds was not strictly correlated with the specific growth rate supported by these compounds when present as the only sources of carbon and energy. Fructose supported only slow growth of *P. aeruginosa* and caused weak repression of ACOAT. Citrate, glutamate and glucose were all good carbon sources, allowing growth rates of approximately 0.8 h⁻¹, but repressed to different extents. Proline was an intermediate carbon source but repressed the synthesis of ACOAT very effectively. The variety of compounds exerting repression of ACOAT (Table 2) suggested that they did not interfere specifically with ACOAT synthesis but were metabolized and thereby led to catabolite repression.

An analysis of the kinetics of citrate-repression of ACOAT revealed some analogies to the phenomenon of catabolite repression as described in the β-galactosidase system of *Escherichia coli* (Magasanik, 1970) and in the amidase system of *P. aeruginosa* (Smyth & Clarke, 1975). Immediately after addition of citrate to a culture growing on arginine plus (NH₄)₂SO₄, the differential rate of ACOAT synthesis went through a phase of severe transient repression which lasted for about two doublings (Fig. 3a). After this period, enzyme synthesis

Table 2. *Repression of ACOAT by carbon sources*

Strain PAO1 was grown in medium P with 15 mM-(NH₄)₂SO₄ and the various additions indicated. ACOAT activities and growth rates were determined as described in Methods.

Addition(s)*	Specific activity of ACOAT [U (mg protein) ⁻¹]	Growth rate constant (h ⁻¹)
Arginine	72.0	0.48
Arginine + Fructose	51.6	0.55
Arginine + Glutamate	43.8	0.80
Arginine + Glucose	34.8	0.80
Arginine + Proline	24.6	0.65
Arginine + Citrate	14.4	0.80
Fructose	4.0	0.25
Glutamate	4.0	0.80
Glucose	4.3	0.80
Proline	3.4	0.55
Citrate	3.2	0.80

* All 20 mM, except glucose and fructose which were added at 28 mM.

Fig. 3. Regulation of the synthesis of ACOAT and aOTC by citrate. Two cultures of strain PAO1 were grown in Fernbach flasks on medium P with 15 mM-(NH₄)₂SO₄ and 20 mM-arginine. For other details, see legend to Fig. 2. The arrow indicates the addition of citrate up to a final concentration of 20 mM to one of the cultures (●); the second culture (■) served as a control. The dashed line represents the steady state rate of enzyme formation on medium P with 15 mM-(NH₄)₂SO₄, 20 mM-arginine and 20 mM-citrate. Differential rates of formation of ACOAT (a) and aOTC (b) were measured in separate experiments.

proceeded at the rate characteristic of the new composition of the growth medium. In contrast to the observations in the amidase system of *P. aeruginosa*, addition of cyclic AMP up to 40 mM relieved neither the transient nor the permanent repression of ACOAT by citrate. The weak repression mediated by 28 mM-glucose or by 20 mM-glutamate was also insensitive to cyclic AMP. Substitution of dibutyryl-cyclic AMP was without effect.

In similar experiments we observed derepression of aOTC by citrate (Fig. 3b).

ACOAT and aOTC are inversely regulated under various growth conditions

There was a linear relationship between the reciprocal of the specific activity of ACOAT and the specific activity of aOTC (Fig. 4) in cells that had been grown in different media. However, in the experiments described so far, the growth rate and thereby the metabolic state of the cells changed from one set of growth conditions to another. It was therefore not clear whether the inverse regulation of ACOAT and aOTC was brought about by a common arginine-specific regulatory system governing the expression of both enzymes and/or by the

Fig. 4. Inverse regulation of the synthesis of ACOAT and aOTC by different carbon sources. Strain PAO1 was grown on medium P with 15 mM-(NH₄)₂SO₄ and 20 mM-arginine. The effect of various additions to this medium on the specific activities of ACOAT and aOTC was determined: 1, no addition; 2, 28 mM-fructose; 3, 20 mM-glutamate; 4, 28 mM-glucose; 5, 20 mM-proline; 6, 20 mM-citrate.

catabolite repression system acting in an opposite manner on the synthesis of the two proteins. We therefore developed a procedure by which the uptake of arginine was restricted without changing the growth rate.

Lysine is a poor growth substrate for *P. aeruginosa* wild type (Fothergill & Guest, 1977). In strain PAO1 it supports a mean generation time of about 11 h when present as the only carbon and nitrogen source in medium P. Lysine also competes with arginine for one of the two transport systems for arginine in *P. putida* (Fan *et al.*, 1972). We could therefore restrict uptake of arginine by adding lysine to the growth medium. The growth rate of strain PAO1 on medium P with arginine and (NH₄)₂SO₄ was progressively inhibited as the concentration of lysine in the medium was increased (Fig. 5). The growth rate of cultures on 20 mM-glutamate, 10 mM-arginine and 15 mM-(NH₄)₂SO₄, however, was not affected by the addition of lysine up to 120 mM. The activities of ACOAT and aOTC determined in cells which had been grown on the latter medium at the same rate but with different lysine concentrations showed inverse regulation of the two enzymes. The enzyme activities depended on the degree of arginine restriction caused by different lysine concentrations during growth (Fig. 6). This experiment suggests that the inverse regulation of ACOAT and aOTC depends on the intracellular concentration of arginine, which might act as a corepressor in a regulatory system controlling the synthesis of the two enzymes. It is not clear at this stage whether carbon sources regulate the expression of the two arginine enzymes indirectly by decreasing the intracellular concentration of arginine or directly by triggering changes in the intracellular concentration of an effector which forms part of a catabolite repression system.

Citrate-resistant mutants

Ten CIT-r derivatives of strain PAO951 were isolated. When they were grown in medium P with ornithine and citrate, their ACOAT activities were 18- to 60-fold higher than the ACOAT activities of the wild type grown under the same conditions. All CIT-r mutants grew extremely slowly on medium P with ornithine and, like strain PAO951, failed to grow on medium P with arginine. Ornithine thus satisfies the proline requirement of strain PAO951 while arginine, although being catabolized via ornithine, is unable to do so. At present we can explain neither the inability of CIT-r mutants to grow on ornithine nor the lack of growth of strain PAO951 on arginine.

One CIT-r mutant, strain PAO959, was compared with the wild type and with its parent,

Fig. 5

Fig. 6

Fig. 5. Inhibition by lysine of growth on arginine. Strain PAO1 was grown in Fernbach flasks on medium P with 15 mM-(NH₄)₂SO₄ and 10 mM-arginine. The effect of increasing concentrations of lysine on the growth rate was determined: ●, no addition; ■, 40 mM-L-lysine; ▲, 80 mM-L-lysine; ○, 120 mM-L-lysine; □, 200 mM-L-lysine.

Fig. 6. Inverse regulation of the synthesis of ACOAT and aOTC by arginine. Strain PAO1 was grown in medium P with 15 mM-(NH₄)₂SO₄, 20 mM-glutamate and 10 mM-arginine. The effect of different degrees of arginine restriction by lysine on the specific activities of ACOAT and aOTC was determined: 1, no addition; 2, 10 mM-L-lysine; 3, 20 mM-L-lysine; 4, 40 mM-L-lysine; 5, 80 mM-L-lysine.

Table 3. *Specific activity of ACOAT and aOTC in the wild type (PAO1), the citrate-resistant mutant (PAO959) and its parent (PAO951) under different conditions of growth*

Growth, preparation of extracts and determination of enzyme activities are described in Methods. The extremely slow growth of strain PAO959 in medium P with ornithine led to the selection of citrate-sensitive revertants, so it was not possible to obtain ornithine-grown cells for enzyme assays. Each value represents the arithmetic mean $\pm$ standard deviation of three independent determinations.

Addition(s)* to medium P	ACOAT activity [U (mg protein) ⁻¹]			aOTC activity [U (mg protein) ⁻¹]		
	PAO1	PAO951	PAO959	PAO1	PAO951	PAO959
Arginine	73.0 $\pm$ 11.0	NG	NG	0.27 $\pm$ 0.10	NG	NG
Glutamate	6.2 $\pm$ 1.1	NG	NG	4.78 $\pm$ 1.41	NG	NG
Proline	5.7 $\pm$ 1.8	5.8 $\pm$ 2.0	66.6 $\pm$ 8.7	2.74 $\pm$ 0.48	5.04 $\pm$ 0.99	0.60 $\pm$ 0.1
Proline + Citrate	5.5 $\pm$ 0.4	4.6 $\pm$ 0.1	83.6 $\pm$ 7.4	3.45 $\pm$ 0.10	5.67 $\pm$ 0.44	0.74 $\pm$ 0.2
Ornithine	34.0 $\pm$ 4.6	13.3 $\pm$ 0.7	—	0.64 $\pm$ 0.24	1.04 $\pm$ 0.17	—
Ornithine + Citrate	11.2 $\pm$ 1.1	NG	120.8 $\pm$ 13.3	2.05 $\pm$ 0.16	NG	2.35 $\pm$ 0.5

NG, No growth. * All 20 mM.

strain PAO951 (Table 3). During growth on medium P with proline, the mutant had an induced level of ACOAT activity and a partially repressed level of aOTC activity whereas strains PAO1 and PAO951 showed non-induced levels of ACOAT and derepressed levels of aOTC. In the CIT-r mutant ACOAT synthesis had become insensitive to repression by citrate while aOTC was still derepressed by the addition of citrate to a medium containing ornithine as the only carbon and nitrogen source but was insensitive to citrate-derepression in the proline medium. Addition of (NH₄)₂SO₄ to the ornithine/citrate medium had no effect on the activity of aOTC in strain PAO959. Thus the mutation to citrate resistance affected the arginine-specific control of ACOAT and aOTC synthesis and at the same time abolished the regulation of the synthesis of ACOAT by citrate. In the absence of a genetic analysis of strain PAO959 it cannot be excluded that the phenotypic properties of the strain are due to a double mutation. However, the high rate at which CIT-r mutants arise and the fact that the ACOAT

and aOTC activities in other CIT-r mutants were similar to the activities in strain PAO959 do not support this possibility. The rate of synthesis of acetamide amidase, another inducible enzyme regulated by catabolite repression (Smyth & Clarke, 1975), was still sensitive to repression by citrate in strain PAO959. As in the wild type the induced rate of amidase synthesis was repressed by approximately 40 % on addition of 10 mM-citrate. It is therefore unlikely that the resistance of ACOAT to citrate repression was due to a change in citrate metabolism leading to a generalized resistance to catabolite repression.

DISCUSSION

Pseudomonas aeruginosa synthesizes arginine from glutamic acid via ornithine and citrulline in eight enzymic steps. The transacetylase modification of the arginine pathway (Fig. 1) provides two enzymes for the formation of ornithine from *N*²-acetylornithine: ornithine acetyltransferase and acetylornithinase. Six of the nine loci coding for the arginine biosynthetic enzymes have been mapped and are scattered in the 18 to 55 min region of the chromosome of strain PAO (Haas *et al.*, 1977). Arginine is also efficiently degraded by *P. aeruginosa*. In contrast to the situation in the biosynthetic pathway, the genetics and the regulation of arginine catabolism are largely unknown. The enzymes present in strain PAO suggest at least two different pathways for the catabolism of arginine: the 'deiminase-pathway' via citrulline, ornithine and glutamate 5-semialdehyde (Fig. 1) and the 'decarboxylase-pathway' leading to agmatine, putrescine and 4-aminobutyrate. In an earlier communication we reported that a mutant lacking ACOAT grows very slowly on arginine and not at all on ornithine (Voellmy & Leisinger, 1976*b*) and therefore concluded that the 'deiminase-pathway' was the preferred route of arginine degradation under the growth conditions we used.

If this is the case, *P. aeruginosa* is faced with the problem of regulating the flow of metabolites participating in opposing pathways. As ornithine and citrulline are intermediates both of arginine biosynthesis and of arginine catabolism, an energy-wasting cycle of arginine breakdown and resynthesis must be avoided during growth on arginine. In *Saccharomyces cerevisiae*, which has a urea cycle, the control of opposing pathways of arginine metabolism has been studied in great detail by Wiame and collaborators (Wiame & Dubois, 1976). They have detected an arginine-specific regulatory system controlling the synthesis of the anabolic and the catabolic urea cycle enzymes in an inverse manner and in addition an inactivation of aOTC by formation of a complex with arginase in the presence of arginine.

The present survey of the regulation of synthesis of eight arginine biosynthetic enzymes in *P. aeruginosa* has confirmed that the synthesis of only two enzymes is effectively regulated by arginine: aOTC, the enzyme processing ornithine in the biosynthetic direction, is repressed about 18-fold by arginine and 7-fold by ornithine, whereas ACOAT, an enzyme which, in addition to its biosynthetic function, catabolizes ornithine to glutamate 5-semialdehyde, is induced 14-fold by arginine and 6-fold by ornithine. Ornithine thus occupies a key position in arginine metabolism and is channelled into either the catabolic or the biosynthetic pathway according to the arginine status of the cell. As arginine and ornithine are interconvertible, we do not know whether both compounds, only one of them, or a metabolite derived from them is the true effector in the regulatory system. One would expect a similar mechanism preventing a futile cycle between citrulline and arginine. Whereas the catabolic ornithine carbamoyltransferase is induced by arginine (Stalon *et al.*, 1972), we lack information on the regulation of argininosuccinate synthetase by arginine. The aOTC of *P. fluorescens* has been shown to form a dead-end complex with citrulline (Stalon *et al.*, 1977). High concentrations of citrulline formed from arginine might thus inhibit aOTC thereby leaving ornithine exclusively accessible to the catabolic reaction under conditions of arginine excess. It is possible that this mechanism and other, as yet unknown, mechanisms acting at the level of enzyme activity also exist in *P. aeruginosa* and contribute to the maintenance of a

balance between arginine synthesis and arginine breakdown under various conditions of growth.

The regulation by carbon sources of the two enzymes whose synthesis is controlled by arginine and ornithine seems physiologically important in the case of ACOAT but is not readily understandable for aOTC. There is no obvious metabolic need to increase the rate of synthesis of an arginine biosynthetic enzyme in the presence of arginine and citrate over the rate observed on arginine alone. The inverse regulation of ACOAT and aOTC by carbon sources could be due to inhibition of arginine uptake by citrate and other carbon sources. This explanation was offered by Isaac & Holloway (1972) who observed that arginine repressed the aOTC of *P. aeruginosa* PAO1 most efficiently when present as the sole carbon source. Alternatively the regulation of the two ornithine-utilizing enzymes by carbon sources might be due to a catabolite repression system which is independent of the arginine regulatory mechanism. In *P. aeruginosa* this type of situation has been observed for two histidine-utilizing enzymes (Potts & Clarke, 1976) and for the aliphatic amidase (Smyth & Clarke, 1975).

Since our physiological experiments did not allow a conclusion as to which one of the two hypotheses was more likely to explain the regulatory phenomena in the arginine system, we isolated mutants (CIT-r) with defective regulation. The properties of one of these, strain PAO959, suggest that one mutational event has completely eliminated the arginine-specific regulation and at the same time has severely altered citrate-regulation of both enzymes. We therefore favour the idea that the inverse regulation of ACOAT and aOTC is due to a specific regulatory system common to both enzymes and that the inducer/corepressor concentration in this system is modulated by citrate and other carbon sources via an entry exclusion mechanism.

The synthesis of several amino acid-catabolizing enzymes has been reported to be governed not only by induction and catabolite repression but also by nitrogen repression. This threefold control of enzyme formation has recently been demonstrated for ACOAT of *Klebsiella aerogenes* (Friedrich *et al.*, 1978) and for histidase and urocanase of *P. aeruginosa* (Potts & Clarke, 1976). In the present study we observed no effect of $(\text{NH}_4)_2\text{SO}_4$ on the repression of ACOAT by citrate. Citrate and other carbon sources repressed the induced synthesis of ACOAT whether $(\text{NH}_4)_2\text{SO}_4$ was present in the medium or not. This observation may either reflect the absence of nitrogen regulation in the control of ACOAT synthesis or it may be due to the generation from the inducer arginine of non-limiting concentrations of ammonium by arginine deiminase and catabolic ornithine carbamoyltransferase. The latter possibility seems likely as the addition of $(\text{NH}_4)_2\text{SO}_4$ had no effect on the growth rate of strain PAO1 on a medium with arginine as the only carbon and nitrogen source. In order to test the involvement of nitrogen repression in the ACOAT/aOTC system it will therefore be necessary to study the regulation of these enzymes under nitrogen limitation in mutants with a defective arginine transport system or defective arginine catabolism.

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