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Catabolic N^2-Acetylornithine 5-Aminotransferase of *Klebsiella aerogenes*: Control of Synthesis by Induction, Catabolite Repression, and Activation by Glutamine Synthetase

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Klebsiella aerogenes formed two N^2-acetylornithine 5-aminotransferases (ACOAT) which were separable by diethylaminoethyl-cellulose chromatography. One ACOAT was repressed when the cells grew on arginine-containing medium, indicating its function in arginine biosynthesis. The second ACOAT was induced when arginine or ornithine was present in the medium as the sole source of carbon or nitrogen, suggesting its function in the catabolism of these compounds. The induced enzyme was purified almost to homogeneity. Its molecular weight is 59,000; it is a pyridoxal 5-phosphate-dependent enzyme and exhibits activity with N^2-acetylornithine (K_m = 1.1 mM) as well as with ornithine (K_m = 5.4 mM). ACOAT did not catalyze the transamination of putrescine or 4-aminobutyrate. The best amino acceptor was 2-ketoglutarate (K_m = 0.7 mM). ACOAT formation was subject to catabolite repression exerted by glucose when ammonia was present in excess. When the cells were deprived of nitrogen, ACOAT escaped from catabolite repression. This activation was mediated by glutamine synthetase as shown by the fact that mutants affected in the regulation or synthesis of glutamine synthetase were also affected in the control of ACOAT formation.

The results presented in the preceding paper indicate that in *Klebsiella aerogenes* arginine is converted to 2-ketoglutarate by a pathway leading through ornithine (9). Cells grown in the presence of arginine were found to contain ornithine aminotransferase (OAT) activity (9). Preliminary experiments showed that this enzyme is more active with N^2-acetylornithine as substrate than with ornithine. Therefore, we had to consider the question of whether this enzyme is identical to the acetylornithine aminotransferase (ACOAT; EC 2.5.1.11) known to catalyze the fourth step in the biosynthesis of arginine in procaryotic and eucaryotic microorganisms (1, 7, 10). The reaction catalyzed by the enzyme, N^2-acetyl-L-ornithine + 2-ketoglutarate $\rightleftarrows$ N^2-acetyl-L-glutamic 5-semialdehyde + L-glutamate, is reversible, and the enzyme might therefore play a role in both arginine degradation and arginine biosynthesis (16).

MATERIALS AND METHODS

Chemicals and enzymes. L-Arginine hydrochloride, L-ornithine hydrochloride, N^2-acetyl-L-ornithine, L-histidine hydrochloride, 1,4-diaminobutane dihydrochloride (putrescine), 4-aminobutyrate, and *o*-aminobenzaldehyde were purchased from Sigma Chemical Co.; L-glutamine was obtained from Calbiochem. Ammonium sulfate, enzyme grade, was a product of Schwarz/Mann; diethylaminoethyl (DEAE)-cellulose (DE-52) was from Whatman; Sephadex G-200 and G-100 were from Pharmacia Fine Chemicals. Proteins for molecular weight determination such as ferritin, rabbit muscle aldolase, ovalbumin, bovine albumin, and chymotrypsinogen A were from Boehringer Mannheim Corp. 2-Methylimidazole and 2,4-dimethylimidazole were purchased from Gallard-Schlesinger and decolorized with Norit before use.

Bacterial strains. The strains of *K. aerogenes* used in this investigation are the same as those described recently (8).

Growth of cells. The minimal salts medium (13) was supplemented with a carbon source to a final concentration of 0.4%; nitrogen sources and glutamine were added to 0.2%; carbon and nitrogenous compounds except ammonium sulfate were sterilized by filtration. Growth conditions for low and high cell mass production are the same as described recently (8).

Preparation of extracts. Cell extracts were prepared by suspending the cells in 0.1 M potassium phosphate buffer, pH 7.0. The cells were subjected to ultrasonic disruption in an MSE sonicator, using 20-s treatments per 0.5 ml of cell suspension at 1.5 A. Cell debris was removed by centrifugation at 50,000 × *g* for 45 min. Protein content of extracts was determined

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by the method of Lowry et al. (11), using bovine serum albumin as a standard. Protein solutions were concentrated in a Diaflo TCF 52 cell (Amicon), using PM 10 membranes. Buffer A, used in the purification of ACOAT, was 50 mM potassium phosphate, pH 7.1, containing 0.5 mM pyridoxal 5-phosphate.

Enzyme assays. One unit of activity is the amount of enzyme that forms 1 μmol of product per min. The specific activity is expressed in units per milligram of protein.

ACOAT was assayed according to Albrecht and Vogel (1). The assay is based on the hydrolysis of the reaction product N^2-acetyl-L-glutamic 5-semialdehyde to L-glutamic 5-semialdehyde, the product of OAT, which cyclizes spontaneously to Δ^1-pyrroline 5-carboxylic acid and reacts with o-aminobenzaldehyde to yield a yellow compound with an absorbance at 440 nm. The reaction mixture contained, in a final volume of 0.5 ml: 100 mM sodium glycinate, pH 9.5; 10 mM N^2-acetyl-L-ornithine; 5 mM 2-ketoglutarate; and 0.5 mM pyridoxal 5-phosphate. Shortly before use the mixture was adjusted to a final pH of 8.9. The reaction was initiated by the addition of 20 μl of enzyme; incubation was carried out at 37°C and terminated by the addition of 0.2 ml of 10 N HCl. The tubes were stoppered and kept for 45 min in a boiling-water bath. After cooling the mixture to room temperature, 1.0 ml of 3.6 M sodium acetate was added followed by 0.2 ml of 10 mM o-aminobenzaldehyde. Color was allowed to develop within 15 min at room temperature. Then the absorbance was read at 440 nm in a Zeiss PMQ II spectrophotometer.

The assay for OAT was the same as that for ACOAT, except that instead of N-acetyl-L-ornithine, 30 mM L-ornithine was added as the substrate and the boiling step was omitted. Both activities were a linear function of the incubation time (up to at least 20 min) and the protein concentration (up to 200 μg/assay). Enzyme activity was calculated using an absorption coefficient for the dihydroquinazolinum product of 1.9×10^3 liter mol^{-1} cm^{-1} at 440 nm as described (16).

The assay for glutamine synthetase as γ-glutamyl transferase has been described (3).

Molecular weight determination. The molecular weight of ACOAT was determined by Sephadex G-200 gel filtration according to the method of Andrews (2). Sephadex G-200 was equilibrated with 50 mM potassium phosphate buffer, pH 7.1, containing 0.5 mM pyridoxal 5-phosphate. The size of the column was 2.5 by 43.5 cm, and fractions of 1 ml were collected. The following standard proteins (molecular weight in parentheses) included in a sample of 0.5 ml of purified ACOAT were identified as follows: ferritin (540,000) by absorbance at 425 nm; catalase (240,000) by following the disappearance of hydrogen peroxide at 240 nm; rabbit muscle aldolase (158,000) by the procedure of Taylor (14); ovalbumin (45,000) and chymotrypsinogen A (25,000) by absorbance at 280 nm. ACOAT was localized according to the standard assay.

Polyacrylamide gel electrophoresis. Analytical electrophoresis was carried out in 7.5% acrylamide gels at 4°C according to Davis (6). For localization of aminotransferase activity, the gels were sliced into 3-mm pieces and incubated with the standard reaction mixture for ACOAT and OAT.

Sodium dodecyl sulfate gels were prepared according to the method of Weber and Osborn (19). The gels were stained for protein with Coomassie brilliant blue.

RESULTS

Separation of catabolic and biosynthetic ACOAT. We found that cells grown on glucose with arginine as source of nitrogen contain 10 to 15 times as much ACOAT as similar cells grown with ammonia as source of nitrogen. We considered the possibility that the ACOAT activity in the ammonia-grown cells is due to an arginine-repressible, biosynthetic enzyme, whereas the ACOAT in the arginine-grown cells reflects the previously discovered arginine-inducible OAT activity (9).

The experiments illustrated in Fig. 1 show

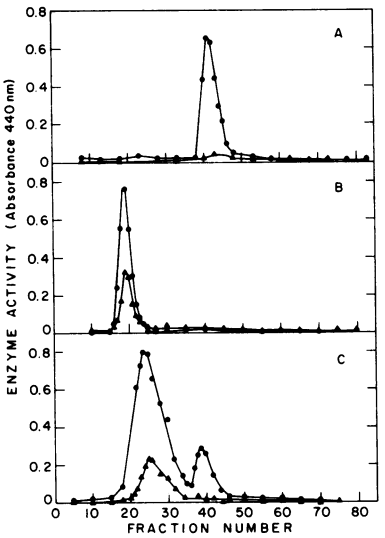

FIG. 1. *Separation of the anabolic and catabolic ACOAT by DEAE-cellulose chromatography. Cells were grown in minimal medium containing 0.4% glucose and 0.2% ammonium sulfate (A) or arginine (B). Six milliliters of each extract (approximately 130 mg of protein) was applied separately to a column of DEAE-cellulose (1.6 by 21 cm) that had been equilibrated with 25 mM potassium phosphate buffer, pH 7.1. Protein was eluted with 0 to 0.5 M linear NaCl gradients prepared in 220 ml of the buffer described above. Fractions of 2.5 ml were collected at a flow rate of 22.5 ml/h. Peak fractions of both runs were pooled, mixed, and applied to another column of DEAE-cellulose (C). Elution was performed as described. Symbols: ●, ACOAT; ▲, OAT. Enzyme assays were carried out under standard conditions. The reaction mixture of (A) contained 50 μl of extract for an incubation period of 20 min. The reaction mixture (B) contained 20 μl of extract for 5 (ACOAT) and 10 (OAT) min. The reaction mixture of (C) contained 50 μl of extract for 10 (ACOAT) and 20 (OAT) min.*

that in arginine- and ammonia-grown cells different proteins are responsible for the respective ACOAT activities. The enzyme of the arginine-grown cells elutes more readily from a DEAE-cellulose column (Fig. 1B) than the enzyme of the ammonia-grown cells (Fig. 1A) and has considerably more OAT activity. An artificially prepared mixture containing both enzymes can be readily separated by chromatography (Fig. 1C).

We may therefore conclude that arginine represses the biosynthetic ACOAT and induces the catabolic ACOAT of *K. aerogenes*.

Purification of catabolic ACOAT. The enzyme was isolated from cells grown on glucose with arginine as sole source of nitrogen. All steps were carried out at 0 to 4°C, and 50 mM potassium phosphate buffer, pH 7.1, containing 0.5 mM pyridoxal 5-phosphate was used to prevent the dissociation of pyridoxal 5-phosphate from the enzyme. The procedure resulting in an 88-fold purification of the enzyme is outlined in Table 1.

It can be seen that the ratio of ACOAT to OAT remained essentially constant during purification, confirming that the two activities are associated with the same protein.

A 50-μg sample of the preparation with the highest specific activity was subjected to nondenaturing electrophoresis. It was found to contain two major bands with both ACOAT and OAT activity and a faint slower-moving band without activity. Electrophoresis on sodium dodecyl sulfate-polyacrylamide gel (denaturing) revealed the presence of a single intensive protein band with a molecular weight of 27,000 and of a faint band corresponding to a molecular weight of 47,000.

The molecular weight of intact ACOAT was estimated by chromatography on Sephadex G-200 as 59,000.

These results suggest that catabolic ACOAT is composed of two identical subunits.

Properties of catabolic ACOAT. Experiments with the 42-fold-purified material (Table 1) indicate that pyridoxal 5-phosphate is the cofactor, since aminooxyacetic acid, a specific inhibitor of pyridoxal 5-phosphate-dependent enzymes (18), at a concentration of 7 mM completely abolished ACOAT activity.

The fact that the enzyme can use both ornithine and N^2-acetylornithine as amino donors suggests strongly that it is a γ-aminotransferase. The enzyme is not active with putrescine and 4-aminobutyrate. The preferred aminoacceptor appears to be 2-ketoglutarate (Table 2). The K_m for 2-ketoglutarate, when acetylornithine or ornithine is added in a concentration sufficient to saturate the enzyme, is 0.7 mM. With 2-ketoglu-

TABLE 2. *Amino acceptors for transferase*

Amino acceptor[a]	Enzyme activity (%)[b]	
	ACOAT	OAT
2-Ketoglutarate	100	100
Pyruvate	6	14
Oxaloacetate	4	10
2-Ketoadipate	68	85

[a] The concentration used was 5 mM.

[b] An enzyme preparation from step 5 of the purification procedure (see Table 1) was used.

TABLE 1. *Purification of ACOAT from K. aerogenes*

Fraction	Vol (ml)	Total protein (mg)	ACOAT (U/mg)	Ratio ACOAT/OAT	Yield (%)	Relative purity
1. Cell extract[a]	33	749	0.39	3.5	100	1.0
2. CTAB[b]	33.5	717	0.43	3.4	107	1.0
3. Ammonium sulfate[c]	4.5	312	0.94	3.9	98	2.4
4. DEAE-cellulose[d]	24	51	4.6	3.7	80	12
5. Sephadex G-200[e]	15	7.9	16.5	3.9	45	42
6. Sephadex G-100[f]	5	1.05	34.6	4.3	12	88

[a] Cells (6.2 g, wet weight) were suspended in buffer A and disrupted sonically; cell debris was removed by centrifugation (50,000 × *g* for 45 min).

[b] Cetyltrimethyl ammonium bromide (CTAB) was added to final concentration of 0.15%, and the mixture was stirred for 15 min; precipitate was removed by centrifugation (10,000 × *g* for 20 min).

[c] Solid ammonium sulfate was added; material precipitating between 30 and 60% saturation (calculated for 25°C) was collected by centrifugation and dialyzed against three 1-liter portions of buffer A.

[d] Residue was applied to a DEAE-cellulose column (1.6 by 24.5 cm) equilibrated with 24 mM potassium phosphate buffer, pH 7.1; protein was eluted with a 0 to 0.5 M linear sodium chloride gradient; fractions (3.0 ml) containing ACOAT activity were pooled and concentrated.

[e] Applied to Sephadex G-200 column (2.5 by 43.5 cm) equilibrated with buffer A and eluted with buffer A; the fraction (3.0 ml) containing the highest ACOAT activity was concentrated and further fractionated.

[f] Concentrated peak fraction contents were applied to a Sephadex G-100 column (1.3 by 50 cm) equilibrated with buffer A; enzyme was eluted with buffer A. The four fractions with greatest ACOAT activity (5.0 ml) were pooled and concentrated.

tarate added in excess, the K_m for acetylornithine is 1.1 mM, and for ornithine it is 5.4 mM. No deviations from classical Michaelis-Menten kinetics were observed.

The enzyme is almost inactive with ornithine as substrate when the pH is below 8.0; the optimum pH is at 9.0. With acetylornithine, the enzyme is active over the wide pH range of 6.5 to 10, with an optimum at 8.7.

The biosynthetic ACOAT, partially purified by DEAE-cellulose chromatography (Fig. 1), is also active between a pH below 7 to a pH of 10, and has maximal activity at a pH of 8.1.

Regulation of synthesis of catabolic ACOAT. The results summarized in Table 3 indicate that formation of the enzyme depends on induction by arginine or ornithine. Cells grown on glucose with ornithine or arginine as source of nitrogen contained approximately 16 times as much ACOAT activity as similar cells grown with ammonia or histidine as source of nitrogen. Considering that the enzyme activity in cells grown in the absence of arginine or, presumably, ornithine reflects the presence of the arginine-repressible biosynthetic ACOAT, the actual ratio of induced to uninduced catabolic ACOAT activity must be much greater than 16.

It is of interest that the high enzyme level found in cells grown with arginine as sole source of carbon and nitrogen is associated with an increased ratio of ACOAT to OAT. When an extract of arginine-grown cells was subjected to chromatography on DEAE-cellulose, the ACOAT activity eluted in the position characteristic for the catabolic enzyme; however, a second minor component not well separated from the bulk of the enzyme could also be discerned. The two components were not completely separated, but all fractions had ACOAT as well as OAT activity (not shown). The altered ACOAT-to-OAT ratio and the altered chromatographic behavior may be accounted for by the presence of either a single enzyme existing in two different polymeric states or two different γ-aminotransferases.

TABLE 3. *Induction of ACOAT*

Composition of medium[a]		Sp act (U/mg)	
Glucose	N-compound	ACOAT	OAT
+	NH_3	0.03	<0.01
+	Arginine	0.48	0.12
−	Arginine	1.05	0.17
+	Ornithine	0.47	0.17
−	Ornithine	0.65	0.21
+	Histidine	0.03	<0.01

[a] The cells were grown in 250 ml of minimal medium supplemented with 0.4% carbon and 0.2% nitrogen sources as indicated. At approximately 100 Klett units, the cells were chilled and washed. Sonic extracts were prepared and assayed for ACOAT and OAT.

The experimental results of Table 4 show that catabolic ACOAT is repressed by glucose and can escape from this repression by starvation for ammonia. The ACOAT level of cells growing on succinate, arginine, and ammonia or on glucose and arginine without ammonia is approximately four times as high as that of cells growing on glucose, arginine, and ammonia. In this respect the regulation of this enzyme resembles that of histidase (13). It has been previously shown that the formation of histidase in glucose-grown cells deprived of ammonia depends on the increased level of glutamine synthetase (12, 15). The experimental results summarized in Table 5 show that glutamine synthetase plays a corresponding role in the regulation of ACOAT formation. For these experiments, the cells were grown in a medium containing glucose, glutamine, and arginine to which ammonia was added where indicated. It is apparent that strain MK-94, whose glutamine synthetase level is high even when it is cultured in an ammonia-containing medium has the same high level of ACOAT whether grown with or without ammonia. Conversely, strain MK-104, which is unable to produce glutamine synthetase, has a low level of ACOAT even when grown without ammonia.

In summary, our results show that the formation of catabolic ACOAT is regulated by induction, catabolite repression, and activation by glutamine synthetase.

DISCUSSION

K. aerogenes produces two N^2-acetylornithine 5-aminotransferases. The enzymes, which can be separated from one another by chromatography, differ to a certain extent in their properties and greatly in the manner of the regulation of their synthesis.

One of the enzymes is repressed by arginine, has maximal ACOAT activity at a pH of 8.1,

TABLE 4. *Catabolite repression of ACOAT*

Composition of medium[a]		Sp act (U/mg)	
C-compound	N-compound	ACOAT	OAT
Glucose	Arginine	0.481	0.119
Glucose	Ammonia + arginine	0.134	0.037
Succinate	Ammonia + arginine	0.547	0.085
Glucose	Ornithine	0.562	0.193
Glucose	Ammonia + ornithine	0.083	0.032

[a] See footnote *a*, Table 3.

TABLE 5. *Effect of glutamine synthetase on the formation of ACOAT*

Strain	Phenotype[a]	Growth medium ± ammonium sulfate[b]	Sp act (U/mg)		
			ACOAT	OAT	GS[c]
MK-53	Gln^+	−	0.312	0.096	0.864
		+	0.145	0.024	0.170
MK-94	GlnC	−	0.404	0.115	0.600
		+	0.412	0.119	0.607
MK-104	Gln^-	−	0.148	0.031	0.0
		+	0.055	0.012	0.0

[a] Gln^+, Glutamine synthetase repressible by ammonia; GlnC, glutamine synthetase not represssible by ammonia; Gln^-, no glutamine synthetase.

[b] See footnote *a*, Table 3. The medium contained glucose, arginine, glutamine, and, where indicated, ammonium sulfate.

[c] GS, Glutamine synthetase.

and has negligible OAT activity. The other enzyme is induced by arginine or ornithine, has maximal ACOAT activity at a pH of 8.7, and has considerable OAT activity.

The arginine-repressible enzyme is presumably responsible for the fourth step in arginine biosynthesis, the conversion of *N*-acetylglutamate 5-semialdehyde to *N*-acetylornithine (1). A similar enzyme of *Escherichia coli* has been described; its loss by mutation results in a requirement for arginine (17).

We have purified the arginine-inducible enzyme to prove that the ACOAT and OAT activities are associated with the same protein moiety. The enzyme has a molecular weight of approximately 59,000 and appears to be composed of two identical subunits.

The catabolic role of the inducible ACOAT is supported by the manner of the regulation of its synthesis. The enzyme is only present at a high level in cells cultured in an arginine- or ornithine-containing medium that lacks either a good energy source, such as glucose, or a good nitrogen source, such as ammonia. This observation indicates that the enzyme is subject to catabolite repression and that its synthesis can be activated by glutamine synthetase (12, 13). This role of glutamine synthetase could be confirmed by the observation that a mutant lacking glutamine synthetase had a low level of ACOAT, even when subjected to ammonia starvation; and that a mutant producing glutamine synthetase constitutively had a high level of ACOAT, even when provided with an excess of ammonia.

The fact that the enzyme is induced by arginine and ornithine is good evidence that it plays a specific role in the degradation of these compounds. Because of the interconvertibility of these compounds we do not know which one is the actual inducer. It is also possible that either one can fulfill this role, or that the true inducer is a metabolite derived from these compounds.

We have provided evidence in the preceding paper that arginine can be converted by *K. aerogenes* to ornithine and eventually to 2-ketoglutarate (9). It is therefore likely that the physiological role of the enzyme depends on its OAT activity, the ability to catalyze the conversion of ornithine to glutamate semialdehyde. In that case it is puzzling that an enzyme designed for this purpose should have greater affinity for acetylornithine than for ornithine and be more active with the former as substrate than with the latter. We cannot exclude at this time the possibility that arginine degradation yields acetylornithine, which is converted to glutamate via *N*-acetylglutamate semialdehyde.

The catabolic ACOAT described in this paper appears to be similar to an ACOAT discovered in a mutant of *E. coli* (5). This mutant was isolated as a revertant to prototrophy of an arginine-requiring mutant lacking the arginine-repressible ACOAT (17). In place of the missing enzyme, it contains an arginine-inducible enzyme with both ACOAT and OAT activity (4).

Another organism possessing arginine-inducible ACOAT with OAT activity is *Pseudomonas aeruginosa* (16). The authors of the paper describing this enzyme consider it to play a role in arginine synthesis as well as in arginine degradation. However, they have not examined the cells grown without arginine in order to discover whether their ACOAT activity reflects the arginine-inducible enzyme at its uninduced level or a separate arginine-repressible enzyme. In view of our present findings, we consider the second possibility the more likely one.

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