Formation and Operation of the Histidine-degrading Pathway in *Pseudomonas aeruginosa*

THOMAS G. LESSIE¹ AND FREDERICK C. NEIDHARDT

Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

Received for publication 7 February 1967

Histidine ammonia lyase (histidase), urocanase, and the capacity to degrade formiminoglutamate, which are respectively involved in steps I, II, and IV in the catabolism of histidine, were induced during growth of *Pseudomonas aeruginosa* on histidine or urocanate, and were formed gratuitously in the presence of dihydro-urocanate. Urocanase-deficient bacteria formed enzymes I and IV constitutively; presumably they accumulate enough urocanate from the breakdown of endogenous histidine to induce formation of the pathway. Urocanate did not satisfy the histidine requirement of a histidine auxotroph, indicating that it probably acted as an inducer without being converted to histidine. The results imply that urocanate is the physiological inducer of the histidine-degrading enzymes in *P. aeruginosa*. Enzymes of the pathway were extremely sensitive to catabolite repression; enzymes I and II, but not IV, were coordinately repressed. Our results suggest a specific involvement of nitrogenous metabolites in the repression. Mutant bacteria with altered sensitivity to repression were obtained. The molecular weight of partially purified histidase was estimated at 210,000 by sucrose gradient centrifugation. Its K_m for histidine was 2×10^{-3} M in tris(hydroxymethyl)aminomethane chloride buffer. Sigmoid saturation curves were obtained in pyrophosphate buffer, indicating that the enzyme might have multiple binding sites for histidine. Under certain conditions, histidase appeared to be partially inactive in vivo. These findings suggest that some sort of allosteric interaction involving histidase may play a role in governing the operation of the pathway of histidine catabolism.

The pathway of histidine catabolism has been studied in *Aerobacter aerogenes* (12) and *Pseudomonas fluorescens* (21), and the individual reactions in the conversion of histidine to glutamate have been elucidated. Studies of the regulation of formation of this pathway have been carried out in *A. aerogenes* (12, 20), *Salmonella typhimurium* (11), and *Bacillus subtilis* (7), but not in the pseudomonads. Where examined, the pathway was found to be inducible and sensitive to catabolite repression. At least for *A. aerogenes*, urocanate, the product of the first enzyme, induces formation of the entire pathway (20). Enzyme formation in response to histidine depends upon conversion of histidine to urocanate. This is possible because low levels of histidase are always present.

The fact that pseudomonads grow well at the expense of a great variety of metabolically diverse carbon compounds suggests that members of this group possess well-developed regulatory

mechanisms. We have studied the regulation of histidine catabolism in *P. aeruginosa* to contribute to comparative studies already in progress, and to gain information about the generality of such regulatory mechanisms as catabolite repression. In particular, we were interested in exploring the possibility that formation of catabolic enzymes is subject to some sort of feedback control involving adenine nucleotides, which might link enzyme formation to the energy demands of the cell.

MATERIALS AND METHODS

Organisms. *P. aeruginosa* ATCC 7700 was used in most of the experiments. Mutant strains derived from strain 7700 are described in the Results section.

Growth conditions. The bacteria were grown in basal salts solution P (5) containing 0.2% (w/v) $(\text{NH}_4)_2\text{SO}_4$, indicated carbon sources (each at 0.3% final concentration), and other supplements as specified. Bacteria were incubated at 37 C. Aerobic cultures were shaken in Erlenmeyer flasks filled to one-fifth capacity. For anaerobic growth, KNO_3 (final concentration, 0.15%) was added to the media, and the cultures were aerated with a gas mixture of 5%

¹ Present address: Department of Microbiology, University of Washington Medical School, Seattle.

(v/v) CO₂ and 95% (v/v) N₂. Growth was monitored by following the increase in optical density of the cultures at 420 m μ . An optical density of 1 was equivalent to 80 μ g of protein per ml.

Mutant screening. Bacteria unable to grow on histidine were obtained by the following procedure. Portions of fully grown succinate cultures of strain 7700 were treated at 37 C with the mutagen *N*-methyl-*N*-nitroso-guanidine (final concentration 100 μ g/ml) for 20 min. The treated samples were diluted in basal salts medium containing 0.2% (NH₄)₂SO₄ and directly plated on solid medium containing succinate as carbon source. Under these conditions, 1 to 5% of the bacteria survived treatment with the mutagen. The survivors were replica-plated to medium containing histidine as the sole carbon source. Plates were screened for colonies failing to grow on histidine. Auxotrophic strains unable to grow on specified amino acids were obtained by a similar procedure. The bacteria were screened by plating on media with and without the amino acid in point.

Chemical determinations. Histidine was assayed by a procedure in which diazotized sulfanilic acid was reacted with the imidazole group of histidine to form a colored product (8). Formiminoglutamate was determined colorimetrically as described by Lund and Magasanik (10). Protein and ribonucleic acid (RNA) were estimated as reported previously (9).

Measurement of in vivo histidine consumption. Portions of culture were filtered through membrane filters (Schleicher and Schuell Co., Keene, N.H.) with a pore size of 0.4 μ . The filtrates were assayed for histidine, and the amount remaining in the medium was plotted as a function of bacterial growth. Disappearance of histidine was expressed as millimicromoles of histidine utilized per milligram of protein formed. This value was converted to millimicromoles of histidine degraded per minute per milligram of protein by multiplying by the appropriate growth rate constant expressed in minutes⁻¹. In these experiments, the amount of histidine consumed per milligram of protein formed was constant over the interval of growth examined, and growth occurred at a constant exponential rate.

Preparation of bacterial extracts for enzyme assays. Cultures were grown to a density equivalent to 80 μ g of protein per ml, and 30- to 100-ml portions were centrifuged. The bacteria were washed with 5×10^{-2} M tris(hydroxymethyl)aminomethane (Tris) chloride (pH 7.4) and suspended in 2 to 3 ml of the same buffer. The suspended cells were disrupted by sonic treatment for 2 min with a Branson 20-kv Sonifier (Branson Instruments Inc., Stamford, Conn.), and the sonically treated material was centrifuged for 20 min at 12,000 $\times g$. The supernatant liquids were decanted and used as a crude source of enzyme.

Enzyme assays. Histidine ammonia lyase (EC 4.3.1.3.), commonly called histidase, was assayed by measuring the formation of urocanate from histidine. This was accomplished by monitoring the increase in absorbance of the reaction mixtures at 277 m μ in a Gilford recording spectrophotometer (Gilford Instruments, Oberlin, Ohio). The results were expressed as millimicromoles of urocanate formed per minute per

milligram of protein, assuming that an increase in absorbance of 1.0 was equivalent to 55.5 μ moles of urocanate formed per ml of reaction mixture. The reaction mixtures (3 ml) contained 5×10^{-2} M Tris chloride buffer (pH 9.2), 2×10^{-3} M reduced glutathione, between 45 and 180 μ g of protein (crude extract), and 10^{-2} M L-histidine (adjusted to pH 9.2 before addition). The reactions were started by addition of histidine.

Urocanase was assayed by measuring the decrease in absorbance at 277 m μ associated with urocanate degradation. The results were expressed as millimicromoles of urocanate consumed per minute per milligram of protein. The assay mixtures (3 ml) contained: 5×10^{-2} M Tris chloride buffer (pH 7.4), between 90 and 300 μ g of protein (crude extract), and 10^{-4} M urocanate. The reactions were started by addition of urocanate. The apparent K_m for urocanate in crude extracts was 8×10^{-5} M. Accordingly, the rates observed under the present conditions represent about half the values expected if the concentration of urocanate had been sufficient to saturate the capacity of the extracts to degrade urocanate.

Degradation of formiminoglutamate was estimated by the same procedure used by Lund and Magasanik to measure formiminoglutamate hydrolase in *A. aerogenes* (10). The reaction mixtures (0.6 ml) contained between 0.25 and 1.0 mg of protein and an initial concentration of 4×10^{-3} M formiminoglutamate. As in the case of *A. aerogenes*, this concentration of formiminoglutamate did not saturate the capacity of the extracts to degrade formiminoglutamate. The assays were carried out at 37 C. Results are expressed as millimicromoles of formiminoglutamate degraded per minute per milligram of protein.

Purification of histidase. Histidine-grown bacteria from a culture containing about 300 μ g of protein per ml were washed in 5×10^{-2} M Tris chloride buffer (pH 7.4) containing 10^{-2} M MgCl₂. The bacteria were suspended in 40 ml of the same buffer containing 2 μ g of deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.) per ml, and were disrupted by passing the suspension through a French pressure cell at between 6,000 and 9,000 psi. The treated material was centrifuged for 20 min at 12,000 $\times g$ to remove unbroken cells. The supernatant liquid (33 ml) was diluted to 40 ml and further centrifuged for 2 hr at 40,000 rev/min in the no. 50 rotor of a Spinco model L preparative ultracentrifuge to remove ribosomes. The supernatant fluid was heated for 20 min at 65 C; denatured protein was removed by centrifugation. Streptomycin sulfate (final concentration, 2%) was added to the preparation to precipitate RNA remaining after the high-speed centrifugation. The precipitate was removed by centrifugation. An equal volume of 50% (w/v) ammonium sulfate was added to the supernatant fluid, and this preparation was incubated for 6 hr at 0 C. The precipitate was collected by centrifugation, and was then dissolved in 5 ml of 5×10^{-2} M Tris chloride buffer (pH 7.4). The enzyme solution was dialyzed against 2 liters of the same buffer. The overall purification was about 10-fold and the recovery was 30%. These steps are outlined in Table 1.

TABLE 1. Purification of histidase from *Pseudomonas aeruginosa*

Fraction	Vol	Protein	Enzyme ^a		Recovery	Purification factor
			Units/ml	Total units		
	ml	mg/ml			%	
1. 12,000 × g	40	45	195	7,800	100	—
2. 40,000 rev/min	32	32	210	6,720	86	1.5
3. 65 C	26	15	210	5,460	70	3.2
4. Streptomycin	28	—	180	5,040	65	—
5. (NH ₄) ₂ SO ₄	5.5	7	390	2,145	28	13

^a In this table, 1 unit is the amount of enzyme that causes an optical density increase of 1.0 per min in the standard assay system.

Sucrose gradient centrifugation. The procedures followed were basically those reported by Martin and Ames (13).

Sephadex gel filtration. Samples (0.5 ml), containing less than 5 mg of protein from a partially purified preparation of histidase and 1 mg of hemoglobin, were loaded on 20 cm by 1 cm² columns of Sephadex G-100 or G-200 gels (Pharmacia Fine Chemicals, Inc., New York, N.Y.) equilibrated with 5×10^{-2} M Tris chloride buffer (pH 7.4), and were eluted with the same buffer. Fractions (1 ml) were collected and assayed for histidase and hemoglobin.

Chemicals. Formiminoglutamate was obtained as the barium salt (Sigma Chemical Co., St. Louis, Mo.), and converted to the sodium form by addition of an equivalent amount of Na₂SO₄. Barium sulfate was removed by centrifugation. Urocanic acid and dihydrourocanic acid were obtained from Calbiochem, Los Angeles, Calif. All other chemicals were of reagent grade.

RESULTS

Induction of histidine degrading enzymes in *P. aeruginosa*. Figure 1 is a schematic representation of the pathway of histidine catabolism in *P. fluorescens* (21). The scheme differs slightly from that of *A. aerogenes*, where formiminoglutamate is cleaved to formamide and glutamate, yielding one less equivalent of NH₃ than the *Pseudomonas* route (12).

We examined extracts of *P. aeruginosa* for the presence of enzymes I, II, and IV. Bacteria grown on histidine or urocanate had all three activities (Table 2). The lower activities of enzymes II and IV compared with that of enzyme I are partially compensated for by the fact that saturating concentrations of substrates were not employed in their determination (see Materials and Methods). When measured in vitro at 37 C, histidase activity was equivalent to 560 mμmoles of urocanate formed per min per mg of protein (twice the value at 25 C), and approximated the in vivo rate of histidine consumption (490 mμmoles of histi-

dine per min per mg of protein). It is interesting to note that *P. aeruginosa* grew almost as rapidly upon urocanate as upon histidine, and that the levels of enzymes were similar in both cases (Table 2). In *A. aerogenes*, growth on urocanate is slower than on histidine, and the activities of enzymes I and II are correspondingly lower (12). An auxotrophic strain of *P. aeruginosa*, 62, which requires histidine for growth, was unable to grow on urocanate unless exogenous histidine was provided. This finding strongly suggests that urocanate acts as an inducer of the enzymes involved in histidine catabolism without being converted to histidine. Dihydrourocanate, which itself did not support growth, was a gratuitous inducer of the histidine-degrading enzymes in *P. aeruginosa*, but the levels of enzymes were lower than with urocanate or histidine (Table 2). In particular, the level of enzyme IV was disproportionately low. The enzymes of the pathway were present in very low (basal) levels during growth on other organic compounds, such as succinate.

A mutant strain, 21, that lacks urocanase activity, was isolated from the wild strain 7700. Mutant 21 formed enzymes I and IV constitutively when grown on glycerol or succinate (Table 3), but the levels of enzymes were lower than in normal bacteria cultivated on urocanate or histidine. The results are analogous to those with a urocanase-deficient strain of *A. aerogenes* (20). Presumably, some endogenous histidine is degraded by basal amounts of histidase, and, with a block in urocanate degradation, the intracellular pool of urocanate rises to a level sufficient to induce extensive formation of the enzymes involved in histidine catabolism. In *A. aerogenes*, histidine itself does not induce formation of enzymes II and IV (20). The present results support the idea that urocanate is the physiological inducer of the histidine-degrading enzymes in *P. aeruginosa*.

Repression of *P. aeruginosa* histidine-degrading enzymes. Enzymes I, II, and IV were repressed in the presence of succinate (Table 4). The repression of enzyme IV was not as great as that of enzymes I and II, which were repressed coordinately.

Further study was concentrated on regulation of histidase formation, because this enzyme was the most readily assayed of the histidine-degrading enzymes, and because we were interested in the possibility that, as the first enzyme in the pathway, it might play a key role in regulating the operation of the entire sequence.

A number of carbon sources besides succinate repressed formation of *P. aeruginosa* histidase. The extent of repression by the different organic compounds corresponded to their ability to pro-

FIG. 1. Pathway of histidine degradation in *Pseudomonas fluorescens* (21).

TABLE 2. Induction of histidine-degrading enzymes

Carbon source ^a	Growth rate constant ^b (hr ⁻¹)	Specific activity ^c					
		I		II		IV	
		Absolute	Relative	Absolute	Relative	Absolute	Relative
Histidine.....	0.69	280	1.00	90	1.00	92	1.00
Urocanate.....	0.64	280	1.00	87	0.97	117	1.19
Glycerol + dihydrourocanate.....	0.20	47	0.17	21	0.23	7	0.09
Succinate.....	0.92	0.6	0.02	<0.5	<0.01	8	0.09

^a All were added at 0.3% final concentration.^b Calculated as ln2/mass doubling time in hours.^c Absolute activities are expressed as units per milligram of protein, where one unit forms or degrades 1 μ mole of urocanate or formiminoglutamate per min. Relative activities are expressed in relation to the value measured in histidine-grown cells, taken as 1.00.

mote rapid growth. The data of Table 5 show in addition that nearly complete repression of the enzyme was obtained in media supplemented with succinate, yeast extract, and Casamino Acids.

A further correlation between growth rate and repressibility was observed in a mutant strain able to grow at only 65% the normal rate on succinate or succinate plus histidine. Strain 51 formed twice as much histidase when grown in media containing histidine and succinate as did its parent (results not shown). This finding is analogous to the observation by Neidhardt (15) that a mutant of *A. aerogenes* which catabolized glucose at less than the normal rate formed more histidase in the presence of glucose than did its parent. In *A. aerogenes*, succinate supports only slow growth and does not repress histidase formation.

Neidhardt suggested that readily catabolized carbon sources give rise to key metabolites which restrict histidase formation. In *A. aerogenes*, synthesis of the repressing metabolites depended upon availability of nitrogen (14). If histidine was the sole source of nitrogen, the enzyme was derepressed even in the presence of glucose. We have observed analogous uncoupling of the repression of enzymes I and II by succinate in *P. aeruginosa* when NH_4^+ salts were omitted from the media. When urocanate was substituted for histidine as the sole source of nitrogen, enzymes I and II were further derepressed (Table 6). Breakdown of urocanate yields one less equivalent of NH_4^+ than does histidine. Accordingly, it was predicted that maintenance of repressing levels of nitrogenous metabolites would demand greater rates of breakdown of urocanate than histidine, and

TABLE 3. Constitutive synthesis of histidine-degrading enzymes by a urocanase-deficient strain

Carbon source ^a	Growth rate constant ^b (hr ⁻¹)	Specific activity ^c					
		I		II		IV	
		Absolute	Relative	Absolute	Relative	Absolute	Relative
Succinate.....	0.90	22	0.08	<0.5	<0.01	30	0.33
Glycerol.....	0.24	125	0.45	<0.5	<0.01	29	0.32
Histidine + glycerol.....	0.24	150	0.54	<0.5	<0.01	72	0.78

^a See footnote a, Table 2.^b See footnote b, Table 2.^c See footnote c, Table 2. The relative values are expressed in relation to the level found in wild-type cells grown on histidine, taken as 1.00.

TABLE 4. Induction of histidine-degrading enzymes in the presence of succinate

Carbon source ^a	Growth rate constant ^b (hr ⁻¹)	Specific activity ^c					
		I		II		IV	
		Absolute	Relative	Absolute	Relative	Absolute	Relative
Histidine + succinate.....	0.92	39	0.14	19	0.21	29	0.32
Urocanate + succinate....	0.95	55	0.20	15	0.17	—	—
Dihydrouracate + succinate.....	0.95	24	0.09	<1	<0.01	—	—

^a See footnote a, Table 2.^b See footnote b, Table 2.^c See footnote c, Table 2.

require greater derepression of the enzymes involved. Presumably, the physiological repressor of enzymes I and II is a nitrogenous compound which is readily formed from succinate.

Several workers have shown that the formation of catabolic enzymes is repressed when bacteria are subjected to a biosynthetic restriction (3, 16). Our results with *P. aeruginosa* confirm these findings. The data of Table 7 show that the levels of histidase in magnesium or methionine-limited chemostat cultures of *P. aeruginosa* were repressed compared with those of batch cultures, and depended upon growth rate. Moreover, when histidase was the limiting carbon source, the level of histidase was slightly derepressed compared with that of batch cultures, and was independent of growth rate (Table 7). The differences in repressed levels between magnesium- and methionine-limited cultures may be related to the overproduction of RNA observed in the case of methionine limitation (Table 7). Further experiments, whose results are not shown here, indicate that *P. aeruginosa* has somewhat relaxed amino acid control over RNA synthesis.

Derepression of histidase formation during anaerobic growth. In view of the known effects of

adenine nucleotides on catabolic enzyme activity (1), we were interested in looking for similar energy-linked control of enzyme formation. This led us to determine the levels of histidase in anaerobic cultures.

P. aeruginosa is able to grow anaerobically at the expense of carbon compounds if nitrate is included in the media as an electron acceptor, but growth is less rapid than under aerobic conditions. It seemed reasonable that the rate of ATP generation in anaerobic cultures was the limiting step in growth.

We measured the levels of histidase in anaerobic cultures provided with histidine as the sole carbon and energy source. Despite the marked decrease in growth rate *vis à vis* aerobic cultures, the anaerobic levels of histidase were derepressed compared with the aerobic values (Table 8). To determine whether derepression of the enzyme was accompanied by a decrease in the growth yield on histidine, we measured the consumption of histidine as a function of bacterial growth. By multiplying the amount of histidine consumed to form 1 mg of bacterial protein by the appropriate growth rate constant, we obtained the specific rate of histidine consumption (Table 9). Although

TABLE 5. *Repression of histidase by readily utilized substrates*

Carbon source ^a	Growth rate constant (hr ⁻¹)	Histidase activity	
		Absolute (units/mg of protein)	Relative
None.....	0.69	280	1.00
Glucose.....	0.85	105	0.38
Glucanate.....	0.85	84	0.30
Pyruvate.....	0.85	135	0.48
Citrate.....	0.92	58	0.21
Glutamate.....	0.92	105	0.38
Oxalacetate.....	0.92	105	0.38
Succinate.....	0.92	39	0.14
Yeast extract (0.1%) + Casamino Acids (0.1%).....	1.05	10	0.04
Yeast extract (0.1%) + Casamino Acids (0.1%) + succinate...	1.05	<1	<0.01

^a All cultures contained L-histidine plus the indicated supplement.

TABLE 6. *Release of catabolite repression of histidase and urocanase in the absence of NH₄⁺*

Additions to basal salts solution ^a	Growth rate constant ^b (hr ⁻¹)	Enzyme activity ^c			
		I		II	
		Absolute	Relative	Absolute	Relative
Histidine.....	0.69	330	1.18	100	1.11
Histidine + succinate.....	0.92	127	0.45	42	0.47
Histidine + succinate + NH ₄ ⁺ ...	0.92	39	0.14	19	0.21
Urocanate + succinate.....	0.95	210	0.75	53	0.59
Urocanate + succinate + NH ₄ ⁺ ...	0.95	55	0.20	15	0.17

^a The supplements were added to basal salts solution P.

^b See footnote b, Table 2.

^c See footnote c, Table 2.

anaerobic cultures contained more histidase than aerobic cultures, they consumed histidine at about one-third the rate of the aerobic cultures, indicating that the rate of histidine consumption was adjusted to match the decrease in growth rate. Compared with aerobic cultures, less than 40% of the histidase in anaerobic cultures was apparently functioning *in vivo* (Table 9).

If the derepressed levels of the enzyme under

TABLE 7. *Repression of histidase during biosynthetically restricted growth*

Limiting nutrient ^a	Growth rate constant ^b (hr ⁻¹)	Histidase activity ^c		RNA/protein (μg/μg)
		Absolute (units/mg of protein)	Relative	
Histidine	0.16	323	1.15	0.21
	0.35	328	1.17	0.27
Magnesium	0.09	84	0.30	0.20
	0.20	110	0.39	0.23
	0.28	132	0.47	0.33
	0.40	166	0.59	0.48
Methionine	0.18	68	0.24	0.59
	0.38	98	0.35	0.86
	0.64	103	0.37	0.80
Unrestricted batch	0.69	280	1.0	0.28

^a Strain 7700 was grown in chemostat cultures limited by the supply of carbon source, histidine (460 μg/ml), or by magnesium (10⁻⁵ M). Strain 60, a methionine auxotroph was limited by the supply of methionine (2.5 μg/ml). The cultures (250 ml) were saturated with O₂ by bubbling air through the medium.

^b See footnote b, Table 2.

^c See footnote c, Table 2.

anaerobic conditions were induced by energy limitation, then it would seem paradoxical that these same conditions led to an equal and opposite control of enzyme activity. It seems more likely that anaerobic growth somehow resulted in partial inactivation of histidase, and that the level of "active" enzyme was then adjusted to match the growth rate. This explanation, however, still does not account for the fact that anaerobic histidase formation was derepressed over the aerobic level when succinate was present.

It should be noted that anaerobic growth caused a partial release of the repression caused by succinate and of the stronger repression promoted by yeast extract and Casamino Acids (Table 8). The fact that anaerobic growth caused a derepression of histidase formation in cultures supplemented with succinate or with yeast extract and Casamino Acids, or with all three, strongly suggests that the *in vivo* decrease in histidase activity during anaerobic growth was not caused by a restriction of the entry of histidine into the cells.

Inhibition of histidase activity during growth with succinate. A further disparity between the *in vivo* rates of histidine utilization and the amounts

TABLE 8. *Derepression of histidase formation during anaerobic growth*

Carbon source ^a	Growth rate constant ^b (hr ⁻¹)	Histidase activity ^c	
		Anaerobic (units/mg of protein)	Anaerobic/aerobic
None.....	0.28	393	1.4
Succinate.....	0.29	87	2.2
Yeast extract (0.1%) + Casamino Acids (0.1%).....	0.40	50	5.0
Yeast extract (0.1%) + Casamino Acids (0.1%) + succinate...	0.40	22	>22.0

^a The supplements were added to histidine minimal medium.

^b See footnote b, Table 2.

^c See footnote c, Table 2. The last column lists the ratio of histidase levels in cultures grown anaerobically in each medium to the corresponding aerobic cultures.

of histidase activity measured in vitro was found in cultures grown in media containing histidine and succinate. Aerobic and anaerobic cultures supplemented with succinate consumed histidine at only 55 and 17%, respectively, of the rates predicted on the basis of their in vitro histidase activity (Table 9). We conclude that the presence of succinate, as well as anaerobic growth, inhibits histidase activity in vivo.

Inhibition of P. aeruginosa histidase by pyrophosphate. Early in our experiments, we observed that assay of histidase activity in crude extracts containing pyrophosphate (a buffer commonly used in studies of this enzyme) was not linear with time. The rate of urocanate formation gradually increased to a maximum, which was usually attained 5 min after addition of histidine. At high concentrations of pyrophosphate (2×10^{-2} M) and low concentrations of histidine (5×10^{-4} M), the enzyme was completely inhibited. If the extracts were dialyzed to remove pyrophosphate, normal enzyme activity was restored, indicating that pyrophosphate did not act by complexing a metal which was loosely bound to the enzyme and essential for activity.

Investigation of the kinetics of saturation of enzyme with histidine in pyrophosphate buffer indicated that pyrophosphate decreased the apparent binding of histidine to the enzyme. It should be noted that Peterkofsky and Mehler (18) observed a similar inhibition of *P. fluorescens* histidase by ethylenediaminetetraacetate (EDTA). We found that EDTA has an analogous effect on

TABLE 9. *Specific rates of histidine consumption in aerobic and anaerobic cultures*

Condition	Carbon source	Growth rate constant	Specific rate of histidine consumption (mg per min per mg of protein)		Apparent active histidase ^b
			Observed	Predicted ^a	
Aerobic	Histidine	hr ⁻¹			%
	Histidine	0.69	0.075	—	100
	Histidine + succinate	0.92	0.006	0.011	55
Anaerobic	Histidine	0.25	0.038	0.105	36
		0.28	0.004	0.023	17

^a Assuming the same rate of histidine consumption for each unit of histidase as in aerobic cultures containing only histidine as carbon source.

^b Calculated as the ratio of the rate of histidine consumption per units of histidase relative to aerobic cultures with histidine as sole carbon source.

P. aeruginosa histidase (results not shown). In our experiments, 10^{-5} M EDTA was as effective as 5×10^{-3} M inorganic pyrophosphate.

Figure 2 shows saturation curves for 10-fold purified *P. aeruginosa* histidase. Results are shown for both Tris chloride and pyrophosphate buffers. Conventional plots of enzyme activity versus histidine concentration gave normal Michaelis kinetics in Tris chloride buffer. On the basis of the reciprocal plot of enzyme activity versus histidine concentration in Tris chloride buffer, a K_m for histidine of 1.8×10^{-3} M was determined. Different results were obtained in pyrophosphate buffers. Here, plots of enzyme activity versus histidine concentration were sigmoid, indicating cooperative binding effects between histidine molecules. The reciprocal plots of enzyme activity versus substrate concentration show this effect quite clearly. As shown in Fig. 2, the apparent strength of binding of histidine to the enzyme was decreased as the concentration of pyrophosphate was increased.

Since sigmoid saturation curves for substrate are characteristic of so-called allosteric enzymes, we have investigated the possibility that *P. aeruginosa* histidase was subject to some sort of feedback inhibition. We tested the following compounds for ability to inhibit the enzyme, but were unable to demonstrate any interaction with the enzyme: citrate, succinate, oxalacetate, glutamate,

FIG. 2. Effect of pyrophosphate on apparent binding of histidine to *Pseudomonas aeruginosa* histidase. Histidase activity was measured as a function of histidine concentration in the presence of $5 \times 10^{-2} M$ Tris chloride buffer (pH 9.2) and different concentrations of pyrophosphate (pH 9.2). The reaction mixtures contained $4 \mu g$ of partially purified histidase. Enzyme activity (v) is expressed as change in optical density at $277 m\mu$ per min. (A) Enzyme activity as a function of histidine concentration. (B) Reciprocal plots of enzyme activity versus histidine concentration.

α -ketoglutarate, phosphoenolpyruvate, glyceraldehyde-3-phosphate, formiminoglutamate, acetate, acetyl-coenzyme A, isocitrate (all at $10^{-3} M$), and adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, guanine triphosphate, and guanine monophosphate (all at $4 \times 10^{-4} M$).

Physical characterization of *P. aeruginosa* histidase. We studied *P. aeruginosa* histidase by sucrose gradient centrifugation and filtration through Sephadex gel columns. The enzyme was completely excluded from G-100 gel and eluted from columns of G-200 gel before hemoglobin. By comparing the sedimentation of histidase and hemoglobin through sucrose gradients, an approximate molecular weight of 210,000 was assigned to the enzyme. Figure 3 shows results with 10-fold purified histidase. In our calculations, we used the same treatment of data as employed by Martin and Ames (13). On the basis of analogous determinations, Hartwell and Magasanik (7) estimated the molecular weight of *Bacillus subtilis* histidase to be 220,000.

Mutant strains of *P. aeruginosa* with altered sensitivity to repression. By screening survivors from cultures of *P. aeruginosa* treated with the mutagen, *N*-methyl-*N*-nitrosoguanidine, we obtained mutant strains which were unable to utilize histidine as their sole source of carbon and energy. One of the mutants, 4, formed lower levels of enzymes I and II when grown in media containing histidine and succinate than did its parent (Table 10). Mutant 4 had lower levels of enzymes I and II when grown in media containing glycerol and histidine than did the previously discussed uro-

FIG. 3. Zone centrifugation of *Pseudomonas aeruginosa* histidase. Samples (0.2 ml) containing 700 μg of partially purified histidase and 200 μg of hemoglobin were layered with 0.2 ml of 4% sucrose as an inverse gradient on 4.8-ml linear gradients of 5 to 20% sucrose containing $5 \times 10^{-2} M$ Tris chloride buffer (pH 7.4). The gradients were centrifuged for 15 hr at 35,000 rev/min in the SW-39 rotor of a Spinco model L preparative ultracentrifuge. The bottoms of the tubes were punctured, and 7-drop fractions were collected. Portions (0.025 ml) of each fraction were assayed for histidase activity (○). The remainder of each fraction was diluted with water, and hemoglobin was determined by measuring the optical density at $405 m\mu$ (□).

canase-deficient strain, 21 (Table 10). The levels of enzyme IV were approximately the same in the two strains. Mutant strain 4 did not grow on urocanate, indicating that the mutation involved more than failure to convert histidine to urocanate. An interesting characteristic of mutant 4 was that histidine increased its growth rate in media containing glycerol (Table 10). Glycerol alone supported only slow growth of the wild strain or of mutant 4 or 21 ($k = 0.25$).

Spontaneous revertants of mutant 4 were selected for their ability to grow on histidine as the sole source of carbon and energy. Several of the revertants were examined for ability to form histidase and urocanase in media containing histidine and succinate. All of these formed higher levels of the enzymes in the presence of succinate than did their parent or the wild strain. Results are shown in Table 10 for two of the revertants, 44 and 47. Both strains had normal levels of enzymes I and II when grown on histidine. The value of enzyme IV was approximately 50% of the wild-type level. The revertants grew well on

TABLE 10. Activity of histidine degrading enzymes in mutant strains with altered sensitivity to repression

Strain	Carbon source ^a	Growth rate constant ^b	Relative activity ^c		
			I	II	IV
		<i>hr</i> ⁻¹			
7700	None	0.69	1.00	1.00	1.00
	Glycerol	0.69	—	—	—
	Succinate	0.92	0.14	0.21	0.32
	Succinate + yeast extract (0.1%) + Casamino Acids (0.1%)	1.05	<0.01	—	—
4	None	0	—	—	—
	Glycerol	0.44	0.05	0.04	0.51
	Succinate	0.97	0.02	0.07	—
44	None	0.60	1.18	—	0.58
	Succinate	0.85	0.86	0.81	0.49
	Succinate + yeast extract (0.1%) + Casamino Acids (0.1%)	0.85	0.12	0.11	—
47	None	0.64	0.97	—	0.63
	Succinate	0.83	0.75	0.69	0.55
	Succinate + yeast extract (0.1%) + Casamino Acids (0.1%)	0.85	0.08	0.09	—

^a See footnote a, Table 2.^b See footnote b, Table 2.^c See footnote c, Table 2.

succinate, and did not form enzymes I or II constitutively.

The histidine-degrading pathway in strains 44 and 47 was not completely insensitive to catabolite repression. When grown in rich medium (Table 10), enzymes I and II were markedly repressed. However, the repression was not as great as in the wild strain (7700) grown under similar conditions (compare the present results with those of Table 5). Apparently, the usual mechanism of repression is altered in the revertant strains.

We obtained histidine saturation curves for histidase from mutant 4 and revertants 44 and 47, and found no difference in K_m compared with that of the enzyme from the wild strain. In each case, the enzyme was inhibited by pyrophosphate, and had the same sedimentation behavior in sucrose gradients as did the enzyme from the wild strain.

DISCUSSION

The present results show that enzymes I and II of the pathway of histidine catabolism in *P. aeruginosa* are extremely sensitive to catabolite repression. Enzyme IV is also repressed, but to a lesser extent than the first two enzymes. Enzymes I and II were coordinately repressed. The possibility that this coordination resulted from the two

activities being properties of one protein was eliminated by separating I and II on the basis of size difference (*unpublished data*).

Our results indicate that urocanate induces formation of the histidine-degrading enzymes, apparently without being converted to histidine. Since a urocanase-deficient strain was internally induced to form these enzymes, it is likely that urocanate is the physiological inducer. Great similarity therefore exists between the physiological control of this pathway in *A. aerogenes* and in *P. aeruginosa*. Furthermore, in both organisms regulation of enzyme formation is consistent with the theory of catabolite repression; glucose is a better substrate for growth than succinate in *A. aerogenes* and is a more effective repressor of histidase; in *P. aeruginosa*, succinate is the better carbon source and is a more effective repressor.

Histidase had sigmoid saturation curves for histidine in the presence of inorganic pyrophosphate. Since it is the first enzyme in the pathway and because such curves are characteristic of so-called allosteric enzymes, the possibility should be considered that histidase may be directly involved in pathway regulation. Sigmoid saturation curves have also been observed with *P. fluorescens* histidase in the presence of EDTA; it has been suggested that binding of histidine to the enzyme

involves interaction with a metal, and that EDTA interferes with this process (18). Since inorganic pyrophosphate, like EDTA, is an effective metal chelator, the action of the two agents may be analogous. Another property of the enzyme which is suggestive of a direct role in pathway regulation is its apparent inactivation during anaerobic growth with nitrate or in the presence of succinate. Similarly, in *A. aerogenes*, biosynthetic restrictions have been observed to decrease the apparent *in vivo* activity of histidase (Neidhardt and Jensen, unpublished data).

One metabolic advantage which might accrue from a mechanism which limits the activity of enzymes involved in histidine catabolism would be to assure preferential utilization of endogenous histidine for biosynthesis whenever an exogenous supply is suddenly depleted. In this regard, it should be noted that the activity of an enzyme involved in the fermentation of another amino acid, threonine, appears to be linked to the energy metabolism of *Clostridium tetanomorphum* (22).

Inhibition of histidase by pyrophosphate might link histidase to the energy demands of the cell. Recently, it has been suggested that pyrophosphate may be directly associated with respiration in bacteria (2). Pyrophosphate is also known to be produced by several monomer activation processes, and is presumably present in the cell whenever the concentration of adenosine triphosphate is high. Thus, pyrophosphate might act as a signal to restrict catabolism whenever the energy supply exceeds the amount necessary for growth. Recently, we have noted that adenosine monophosphate and guanine diphosphate antagonize the effects of pyrophosphate (unpublished data). If these events occur in the cell, they would result in increased histidase activity in the presence of metabolites that signal inadequate energy supply, and decreased activity when the supply is abundant.

This model satisfactorily accounts for the fact that succinate, which had no effect on histidase activity *in vitro*, appeared to inhibit histidase *in vivo*; the rapid metabolism of succinate would be expected to saturate the energy requirements of the cell. The model fails, however, to account for the apparent inhibition of histidine metabolism during anaerobic growth with nitrate. Furthermore, the levels of pyrophosphate required for inhibition of histidase *in vitro* seem too high to be achieved *in vivo*.

Another possible regulatory role of histidase is suggested by the fact that urocanate, the product of the first enzyme, seems to be the inducer of the pathway. Induction by the first intermediate in a pathway appears to occur in the case of the degradative pathways for histidine in *A. aerogenes*

(20) and for tryptophan in *P. fluorescens* (17), and the biosynthetic pathway for leucine in *Neurospora* (6). "Repression" of such routes might actually involve restricted formation of inducer as a result of inhibition of the first enzyme. If so, repression by succinate should be effective against induction by histidine, but not against induction by urocanate or dihydrourocanate. In fact, succinate causes catabolite repression in both cases. Therefore, this hypothesis cannot be correct, unless succinate impedes the entry of urocanate and dihydrourocanate into the cell.

If the properties of histidase are related to some participation of the enzyme in repression, either by controlling urocanate formation or by interacting with repressing metabolites while it is still a nascent chain, then one would expect to isolate some regulatory mutants with an altered histidase. We examined the histidase of mutants of *P. aeruginosa* with modified sensitivity to repression to see whether the changes in repressibility were associated with altered histidase. The histidase of mutant 4 and the revertant strains 44 and 47 had the same sedimentation behavior on sucrose gradients, the same sensitivity to pyrophosphate repression, and the same K_m in Tris chloride buffer as did the enzyme of the wild strain.

In summary, we have found that the enzymes of the histidine-degrading pathway are induced by urocanate, and are sensitive to catabolite repression. Although histidase had sigmoid kinetics in the presence of pyrophosphate, and under certain conditions appeared to be partially inactive *in vivo*, we found no direct evidence for a role of the enzyme in pathway regulation.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant GM-08437 from the Division of General Medical Sciences. The senior author held a Public Health Service postdoctoral fellowship (5-F2-AI-16,984-03) from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. ATKINSON, D. E. 1965. Biological feedback control at the molecular level. *Science* **150**:851-857.
2. BALTSCHIEFFSKY, H., L. V. VON STEDINGK, H. W. HELDT, AND M. KLINGENBERG. 1966. Inorganic pyrophosphate: formation in bacterial photophosphorylation. *Science* **153**:1120-1122.
3. CLARK, D. J., AND A. G. MARR. 1964. Studies on repression of β -galactosidase in *Escherichia coli*. *Biochim. Biophys. Acta* **92**:85-98.
4. COHEN, G. N. 1965. Regulation of enzyme activity in microorganisms. *Ann. Rev. Microbiol.* **19**: 105-126.
5. FRAENKEL, D. G., AND F. C. NEIDHARDT. 1961.

- Use of chloramphenicol to study control of RNA synthesis in bacteria. *Biochim. Biophys. Acta* **53**:96-110.
6. GROSS, S. R. 1965. The regulation of synthesis of leucine biosynthetic enzymes in *Neurospora*. *Proc. Natl. Acad. Sci. U.S.A.* **54**:1538-1546.
 7. HARTWELL, L. H., AND B. MAGASANIK. 1963. The molecular basis of histidase induction in *Bacillus subtilis*. *J. Mol. Biol.* **7**:401-420.
 8. JORPES, E. 1932. The colorimetric determination of histidine. *Biochem. J.* **26**:1507-1511.
 9. LESSIE, T. G. 1965. RNA metabolism of *Rhodospseudomonas spheroides* during preferential photopigment synthesis. *J. Gen. Microbiol.* **41**:37-45.
 10. LUND, P., AND B. MAGASANIK. 1965. N-Formimino-L-glutamate formiminohydrolase of *Aerobacter aerogenes*. *J. Biol. Chem.* **240**:4316-4319.
 11. MAGASANIK, B. 1963. The genetic and molecular basis of catabolite repression, p. 271-286. *In* H. J. Vogel, V. Bryson, and J. O. Lampen [ed.], *Informational macromolecules*. Academic Press, Inc., New York.
 12. MAGASANIK, B., P. LUND, F. C. NEIDHARDT, AND D. T. SCHWARTZ. 1965. Induction and repression of the histidine degrading enzymes in *Aerobacter aerogenes*. *J. Biol. Chem.* **240**:4320-4324.
 13. MARTIN, R. G., AND B. N. AMES. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* **236**:1372-1379.
 14. NEIDHARDT, F. C., AND B. MAGASANIK. 1957. Reversal of the glucose inhibition of histidase biosynthesis in *Aerobacter aerogenes*. *J. Bacteriol.* **73**:253-259.
 15. NEIDHARDT, F. C. 1960. Mutant of *Aerobacter aerogenes* lacking glucose repression. *J. Bacteriol.* **80**:536-543.
 16. NEIDHARDT, F. C. 1965. Role of enzyme repression in the regulation of catabolism in bacteria, p. 329-336. *In* *Mécanismes de régulation des activités cellulaires chez les microorganismes*. Colloq. Intern. Centre Natl. Rech. Sci. (Paris) No. 124.
 17. PALLERONI, N. J., AND R. Y. STANIER. 1965. Regulatory mechanisms governing synthesis of the enzymes for tryptophan oxidation by *Pseudomonas fluorescens*. *J. Gen. Microbiol.* **35**:319-334.
 18. PETERKOFKY, A., AND L. N. MEHLER. 1963. Inhibition of pseudomonas histidase: Evidence for a metal cofactor. *Biochim. Biophys. Acta* **73**:159-162.
 19. SCHLESINGER, S., AND B. MAGASANIK. 1965. Imidazole propionate, a nonmetabolizable inducer for the histidine degrading enzymes in *Aerobacter aerogenes*. *J. Biol. Chem.* **240**:4325-4330.
 20. SCHLESINGER, S., P. SCOTTO, AND B. MAGASANIK. 1965. Exogenous and endogenous induction of the histidine degrading enzymes in *Aerobacter aerogenes*. *J. Biol. Chem.* **240**:4331-4337.
 21. TABOR, H. 1955. Degradation of histidine, p. 373-390. *In* W. D. McElroy and H. B. Glass [ed.], *Amino acid metabolism*. Johns Hopkins Press, Baltimore.
 22. TOKUSHIGE, M., H. R. WHITELEY, AND O. HAYASHI. 1963. Energy-linked regulation of threonine metabolism by ADP. *Biochem. Biophys. Res. Commun.* **13**:380-385.