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Feedback Regulation of Arginine Biosynthesis in Blue-Green Algae and Photosynthetic Bacteria

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ABSTRACT

HOARE, D. S. (The University of Texas, Austin), AND S. L. HOARE. Feedback regulation of arginine biosynthesis in blue-green algae and photosynthetic bacteria. J. Bacteriol. **92**:375-379. 1966.—A number of blue-green algae and photosynthetic bacteria synthesize arginine from glutamate via acetylated intermediates. Cell-free extracts of these photosynthetic microorganisms contain an *N*-acetyl glutamate phosphokinase, which is specifically inhibited by arginine. They also contain a transacetylase which forms ornithine from αN-acetyl ornithine and glutamate. The transacetylase appears to be specific for L-glutamate. Arginine synthesis and its regulation by feedback inhibition in photosynthetic microorganisms differ from that in *Escherichia coli* and other *Enterobacteriaceae*.

It was previously reported that some autotrophic blue-green algae can photoassimilate acetate and incorporate it into four amino acids of the cell proteins: namely glutamic acid, proline, arginine, and leucine (6). In examining cell-free extracts of the blue-green alga *Anacystis nidulans* for enzyme activities involved in the biosynthesis of these amino acids from acetate, we have found that glutamate is converted to ornithine via acetylated derivatives, and that one enzyme in this pathway, *N*-acetyl glutamate phosphokinase, is specifically inhibited by arginine. Ornithine is formed from αN-acetyl ornithine by a specific transacetylase which was first described in *Micrococcus glutamicus* (13), and subsequently was demonstrated in yeast (3). A survey of a number of blue-green algae and photosynthetic bacteria has shown that all of these organisms contain the transacetylase and an arginine-sensitive *N*-acetyl glutamate phosphokinase. These findings supplement the recent survey of a number of nonphotosynthetic bacteria by Udaka (12).

MATERIALS AND METHODS

Blue-green algae were grown autotrophically in the light at 30 C on the medium D_M of Van Baalen (14). Cultures were grown in 2-liter batches aerated continuously with a stream of 1 to 2% carbon dioxide in air. Most of the purple nonsulfur photosynthetic bacteria were grown semianaerobically in the light at 30 C in completely filled Roux bottles on a malate plus yeast extract medium (5). Pigment-deficient mutants of *Rhodopseudomonas spheroides* and *Rhodospirillum molischianum* were grown on the malate plus yeast extract medium anaerobically in the light under an atmosphere of 95% hydrogen plus 5% carbon dioxide. *Rhodomicrobium vannielli* was grown anaerobically under 95% hydrogen plus 5% carbon dioxide on the medium of Duchow and Douglas (4) with acetate as major carbon source. Chromatium D and Thiopedia were grown autotrophically as described by Hurlbert and Lascelles (7). *Chlorobium thiosulphatophilum* was grown autotrophically on the medium of Larsen (8), and *Chloropseudomonas ethylicum* was grown anaerobically in the light with acetate as major carbon source (2). All organisms were harvested in the logarithmic phase of growth and were washed twice with 0.05 M KH_2PO_4/KOH buffer (*p*H 7.0).

Cell-free extracts were prepared as follows: 2 to 3 g (wet weight) of packed cells was mixed with 10 g of washed fine glass beads and suspended in 15 ml of 0.05 M KH_2PO_4/KOH (*p*H 7.0). Cell suspensions were disrupted by sonic treatment for 10 min in an M.S.E. sonic disintegrator (Measuring and Scientific Equipment, Ltd., London, England) with a titanium probe 0.5 inch (1.27 cm) in diameter. Disintegrated cell suspensions were centrifuged for 20 min at 25,000 $\times$ *g* and then for 45 min at 100,000 $\times$ *g*. Protein concentrations of the extracts were determined from the absorbance at 260 and 280 mμ (19).

N-Acetyl glutamate phosphokinase activity was determined by the hydroxamate procedure. Reaction mixtures with a total volume of 1.5 ml contained (micromoles): adenosine triphosphate (ATP), 10; $MgCl_2$, 10; *N*-acetyl glutamate, 20; KH_2PO_4 buffer (*p*H 7.5), 100; freshly neutralized hydroxylamine hydrochloride, 200; and extracts containing 2 to 3 mg of protein. After 30 min of incubation at 35 C, 1.5 ml of ferric chloride reagent was added, the mixture was centrifuged, and the absorbance of the

supernatant fluid was read at 540 mμ against a ferric chloride reagent blank. Controls were included in which *N*-acetyl glutamate was omitted.

Transacetylation was demonstrated in cell-free extracts as follows. Reaction mixtures with a total volume of 0.5 ml contained (micromoles): *N*-acetyl glutamate, 10; DL-ornithine, 10; KH_2PO_4 buffer (*p*H 7.0), 20; and extract (2 to 5 mg of protein). Controls were included in which the substrates were omitted singly and together. After 60 min of incubation at 35 C, 10 μliters of 60% perchloric acid was added, and the tubes were centrifuged. The supernatant fluids were decanted, a drop of phenol red indicator was added, and the tube contents were neutralized with 4 N KOH. The tubes were chilled on ice and then centrifuged. 50 μliters of the clear supernatant solutions was then applied to 3 MM paper and subjected to high-voltage electrophoresis at *p*H 3.6 for 2 hr at 27 to 30 v/cm (6). Dried papers were developed by dipping in 0.01% ninhydrin in acetone and heating for 3 to 5 min at 100 C. Electrophoresis under these conditions effects a good separation of the four reactants. The transacetylation reaction is freely reversible, but it has been found more convenient to follow it with *N*-acetyl glutamate as substrate, since the two products α*N*-acetyl ornithine and free glutamate can be detected with ninhydrin. When the reaction is followed with α*N*-acetyl ornithine as substrate, only one of the products (ornithine) can be detected with ninhydrin, and in some cases this might also arise by deacetylation. When the reaction was followed with α*N*-acetyl ornithine as substrate, the reaction mixture contained, in a total volume of 0.5 ml (micromoles): α*N*-acetyl ornithine, 10; glutamate, 10; KH_2PO_4 buffer (*p*H 7.0), 20; and extract (2 to 3 mg of protein); controls were run in which glutamate was omitted.

Results and Discussion

Using the above procedures, we examined a limited number of photosynthetic microorganisms for the presence of the transacetylase and *N*-acetyl glutamate phosphokinase. We also assayed the latter enzyme in the presence of 5, 2, 1, and 0.5 μmoles of L-arginine to determine its sensitivity to inhibition by arginine. The results are shown in Table 1. All the photosynthetic microorganisms so far examined contain the transacetylase and an arginine-sensitive *N*-acetyl glutamate kinase.

Studies on the *N*-acetyl glutamate kinase of *Anacystis nidulans* have shown that other amino acids do not significantly inhibit activity. Inhibition by arginine appears to be partially competitive, since inhibition by a given concentration of arginine is overcome by increasing the substrate concentration (Table 2). Purification of the enzyme is necessary before one can determine more precisely the nature of the inhibitory action of arginine. *A. nidulans* appears to synthesize ornithine exclusively by transacetylation, since extracts of *A. nidulans* did not deacetylate α*N*-acetyl ornithine whether or not cobalt salts or glutathione, or both, were added (17).

Qualitative tests demonstrated that the transacetylation reaction was highly specific for L-glu-

TABLE 1. *N-acetyl glutamate phosphokinase (NAG-kinase) and transacetylase in blue-green algae and photosynthetic bacteria*

Organism	NAG kinase[a]	Per cent inhibition by arginine (μmoles)				Transacetylase
		5	2	1	0.5	
Blue-green algae						
Anacystis nidulans	.10	100	100	86	70	++
Anabaena variabilis	.08	100	100	95	70	++
Nostoc muscorum G	.21	100	100	96	50	++
Synechococcus sp	.08	80	67	35	12	++
Photosynthetic bacteria						
Rhodospirillum rubrum	.10	92	80	60	25	++
R. molischianum	.11	85	70	54	36	++
Rhodopseudomonas capsulata	.17	96	85	60	38	++
R. gelatinosa	.12	90	80	60	40	++
R. palustris	.15	90	80	67	30	++
R. spheroides	.10	65	50	40	15	++
R. spheroides G1 (mutant)	.16	80	60	40	25	++
R. spheroides 2, 4, 1 GA (mutant)	.14	86	70	47	25	++
Rhodomicrobium vannielii	.20	96	90	85	30	++
Chromatium D	.12	100	100	97	55	++
Thiopedia sp	.05	85	65	25	—	++
Chlorobium thiosulphatophilum	.13	100	100	96	73	++
Chloropseudomonas ethylicum	.07	100	100	92	77	++

[a] Expressed as optical density at 540 mμ per hour per milligram of protein.

TABLE 2. *Effect of substrate concentration on the inhibition of the N-acetyl glutamate phosphokinase of Anacystis nidulans by arginine*[a]

N-acetyl glutamate (*μmoles*)	Optical density at 540mμ in tubes with L-arginine (μmoles)		
	0	0.5	1.0
5	.103	.014	.0
10	.140	.039	.020
20	.164	.130	.063
40	.173	.178	.128
60	.193	.195	.148

[a] Reaction mixture (1.5 ml) contained: 2.9 mg of protein, 100 μmoles of KH_2PO_4 (pH 7.5), 10 μmoles of $MgCl_2$, 10 μmoles of ATP, 200 μmoles of NH_2OH, and substrate as indicated. Incubation was for 30 min at 35 C.

tamate. When the reaction was followed with α*N*-acetyl ornithine as substrate, no transacetylation was found with D-glutamate, L-glutamine, L-aspartate, or L-threo-β-methyl aspartate.

This survey of the presence of a transacetylase and an arginine-sensitive *N*-acetyl glutamate kinase supplements the recent report by Udaka (12) on the distribution of these enzymes in bacteria. Bacteria appear to fall into two groups on the basis of the enzyme steps leading from glutamate to ornithine via acetylated derivatives (see Fig. 1, schemes A and B). In scheme A, ornithine is formed from α*N*-acetyl ornithine by a deacetylase which is activated by cobalt and glutathione (17). In *Escherichia coli*, arginine does *not* inhibit *N*-acetyl glutamate phosphokinase (12). Studies with cell suspensions of a mutant of *E. coli* which is blocked in the conversion of *N*-acetyl glutamate to *N*-acetyl glutamic γ-semialdehyde, have established that arginine specifically inhibits the initial reaction involving the synthesis of *N*-acetyl glutamate (18). The enzyme catalyzing the synthesis of *N*-acetyl glutamate from acetyl coenzyme A and glutamate has not been extensively studied in other bacteria. This mechanism is probably used by the *Enterobacteriaceae*, since *Aerobacter aerogenes*, *Serratia marcescens*, and *Proteus vulgaris* also contain the deacetylase and an arginine-insensitive *N*-acetyl glutamate phosphokinase (12); we have obtained similar results with *Salmonella typhimurium*, *Shigella flexneri*, *Erwinia carotovora*, Providence B, and *Proteus morganii*. In scheme B, ornithine is formed from α*N*-acetyl ornithine by transacetylation with glutamate, so that there is a cyclic series of reactions in which *N*-acetyl glutamate is required in only catalytic amounts. Hence, inhibition of the formation of *N*-acetyl glutamate would not control the synthesis of arginine. Arginine specifically inhibits *N*-acetyl glutamate phosphokinase, thus regulating the cyclic process. This scheme operates in the photosynthetic bacteria and blue-green algae and in some other gram-positive and gram-negative bacteria. Evidence in support of a "nonacetylated" pathway in *Neurospora crassa* and some other microorganisms was marshalled by Vogel and co-workers (11, 16), but to date there is no conclusive evidence establishing all the steps leading from glutamate to ornithine by a "nonacetylated" pathway in microorganisms.

Proline is also derived from glutamate, at least in *Escherichia coli* (1, 10, 15) and probably also in the blue-green alga *Anacystis nidulans* (6). Although proline can be synthesized from *N*-acetyl glutamate via ornithine in *E. coli*, this appears not to be the major pathway in vivo (10). Proline biosynthesis in *E. coli* proceeds by a "non-

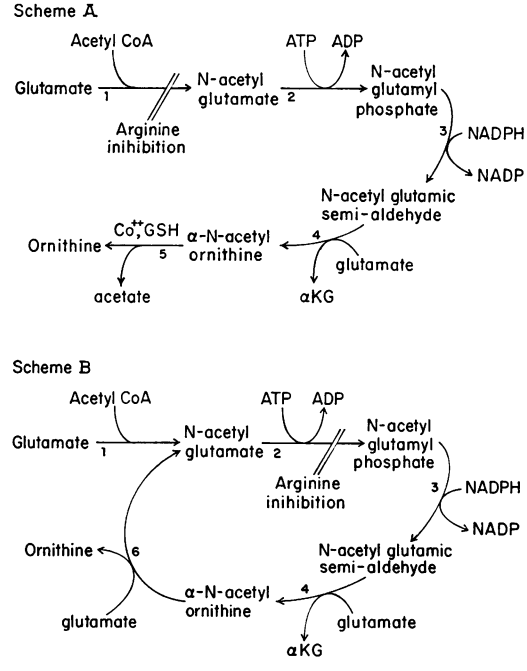

FIG. 1. *Enzymatic pathways in the formation of ornithine from glutamate. Enzymes involved: (1) N-acetyl glutamate synthetase, (2) N-acetyl glutamate phosphokinase, (3) N-acetyl glutamyl γ-phosphate-nicotinamide adenine dinucleotide reductase, (4) αN-acetyl ornithine δ-transaminase, (5) αN-acetyl ornithine deacetylase, and (6) N-acetyl glutamate-ornithine transacetylase. ATP = adenosine triphosphate; ADP = adenosine diphosphate; NADP = nicotinamide adenine dinucleotide phosphate; NADPH = reduced NADP; αKG = α-ketoglutarate; GSH = glutathione.*

TABLE 3. *Effects of arginine and proline on hydroxamate formation from glutamate and N-acetyl glutamate by extracts of Anacystis nidulans, Rhodomicrobium vannielii, and Rhodopseudomonas palustris*[a]

Organism	Protein (mg)	Substrate	Inhibitor	Optical density at 540 mμ
Anacystis nidulans	2.7	*N*-acetyl glutamate	None	.127
			Arginine	.023
			Proline	.129
		Glutamate	None	.059
			Arginine	.070
			Proline	.059
Rhodomicrobium vannielii	3.4	*N*-acetyl glutamate	None	.170
			Arginine	0
			Proline	.161
		Glutamate	None	.093
			Arginine	.073
			Proline	.073
Rhodopseudomonas palustris	3.2	*N*-acetyl glutamate	None	.232
			Arginine	.023
			Proline	.260
		Glutamate	None	.200
			Arginine	.200
			Proline	.207

[a] Reaction mixture (1.5 ml) contained (micromoles): KH_2PO_4 (*p*H 7.5), 100; $MgCl_2$, 10; ATP, 10; NH_2OH, 200; substrate, 20; inhibitor, 20; and cell-free extract. Incubation was for 30 min at 35 C.

acetylated route" via glutamic γ-semialdehyde, and recent studies on growing and resting cells have established that proline inhibits the reduction of glutamate to glutamic γ-semialdehyde (1). By analogy with the formation of *N*-acetyl glutamic γ-semialdehyde from *N*-acetyl glutamate, it is probable that the reduction of glutamate to glutamic γ-semialdehyde proceeds via glutamyl γ-phosphate. Feedback regulation of proline biosynthesis may therefore operate by inhibition of a glutamate phosphokinase or by inhibition of the reduction of glutamyl γ-phosphate to glutamic γ-semialdehyde. As shown in Table 3, extracts of the blue-green alga *A. nidulans* and of two of the photosynthetic bacteria which we have examined do form a hydroxamate from glutamate under conditions similar to those used for the assay of *N*-acetyl glutamate kinase. However, the reaction proceeded much more slowly and was not inhibited by L-proline or L-arginine. It is possible that proline synthesis in these photosynthetic microorganisms is regulated by inhibition of the reduction of glutamyl-γ-phosphate. Extracts of all the photosynthetic microorganisms were found to form a hydroxamate from glutamate, but this activity may be due to the action of glutamine synthetase (9). The problem of the regulation of proline synthesis in photosynthetic microorganisms is still unresolved.

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LITERATURE CITED

1. BAICH, A., AND D. J. PIERSON. 1956. Control of proline biosynthesis in *Escherichia coli*. Biochim. Biophys. Acta **104**:397–404.
2. BOSE, S. K. 1963. Media for anaerobic growth of photosynthetic bacteria, p. 501–510. *In* H. Gest, A. san Pietro, and L. P. Vernon [ed.], Bacterial photosynthesis. The Antioch Press, Yellow Springs, Ohio.
3. DE DECKEN, R. H. 1963. Biosynthèse de l'arginine chez la levure. I Le sort de la N^{α}-acetyl ornithine. Biochim. Biophys. Acta **78**:606–616.
4. DUCHOW, E., AND H. C. DOUGLAS. Rhodomicrobium vannielii, a new photoheterotrophic bacterium. J. Bacteriol. **58**:409–416.
5. HOARE, D. S. 1963. The photoassimilation of ace-

tate by *Rhodospirillum rubrum*. Biochem. J. **87**:284–301.
6. HOARE, D. S., AND R. B. MOORE. 1965. Photoassimilation of organic compounds by autotrophic blue-green algae. Biochim. Biophys. Acta **109**:622–625.
7. HURLBERT, R. E., AND J. LASCELLES. 1963. Ribulose diphosphate carboxylase in Thiorhodaceae. J. Gen. Microbiol. **33**:445–458.
8. LARSEN, H. 1952. On the culture and general physiology of the green sulfur bacteria. J. Bacteriol. **64**:187–196.
9. RAVEL, J. M., J. S. HUMPHREYS, AND W. SHIVE. 1965. Control of glutamine synthesis in *Lactobacillus arabinosus*. Arch. Biochem. Biophys. **111**:720–726.
10. REED, D. E., AND L. N. LUKENS. 1966. Observations on the conversion of N-acetyl glutamate to proline in extracts of *Escherichia coli*. J. Biol. Chem. **241**:264–270.
11. SHER, W. I., AND H. J. VOGEL. 1957. Occurrence of ornithine δ-transaminase: a dichotomy. Proc. Natl. Acad. Sci. U.S. **43**:796–803.
12. UDAKA, S. 1966. Pathway-specific pattern of control of arginine biosynthesis in bacteria. J. Bacteriol. **91**:617–621.
13. UDAKA, S., AND S. KINOSHITA. 1958. Studies on L-ornithine fermentation. I. The biosynthetic pathway of L-ornithine in *Micrococcus glutamicus*. J. Gen. Appl. Microbiol. (Tokyo) **4**:272–282.
14. VAN BAALEN, C. 1965. Quantitative surface plating of coccoid blue-green algae. J. Phycol. **1**:19–22.
15. VOGEL, H. J. 1955. On the glutamate-proline-ornithine interrelation in various microorganisms, p. 335–346. *In* W. D. McElroy and B. Glass [ed.], Amino acid metabolism. Johns Hopkins Press, Baltimore.
16. VOGEL, H. J., AND D. M. BONNER. 1954. On the glutamate-proline-ornithine interaction in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **40**:688–692.
17. VOGEL, H. J., AND D. M. BONNER. 1956. Acetyl ornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**: 97–106.
18. VYAS, S., AND W. K. MAAS. 1963. Feed back inhibition of acetyl glutamate synthetase by arginine in *Escherichia coli*. Arch. Biochem. Biophys. **100**:542–546.
19. WARBURG, O., AND W. CHRISTIAN. 1942. Isolierung und kristallization des Gärungsferments Enolase. Biochem. Z. **310**:384–421.