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Regulation of Neurotoxin and Protease Formation in *Clostridium botulinum* Okra B and Hall A by Arginine

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Supplementation of a minimal medium with high levels of arginine (20 g/liter) markedly decreased neurotoxin titers and protease activities in cultures of *Clostridium botulinum* Okra B and Hall A. Nitrogenous nutrients that are known to be derived from arginine, including proline, glutamate, and ammonia, also decreased protease and toxin but less so than did arginine. Proteases synthesized during growth were rapidly inactivated after growth stopped in media containing high levels of arginine. Separation of extracellular proteins by electrophoresis and immunoblots with antibodies to toxin showed that the decrease in toxin titers in media containing high levels of arginine was caused by both reduced synthesis of protoxin and impaired proteolytic activation. In contrast, certain other nutritional conditions stimulated protease and toxin formation in *C. botulinum* and counteracted the repression by arginine. Supplementation of the minimal medium with casein or casein hydrolysates increased protease activities and toxin titers. Casein supplementation of a medium containing high levels of arginine prevented protease inactivation. High levels of glucose (50 g/liter) also delayed the inactivation of proteases in both the minimal medium and a medium containing high levels of arginine. These observations suggest that the availability of nitrogen and energy sources, particularly arginine, affects the production and proteolytic processing of toxins and proteases in *C. botulinum*.

Botulinum toxin is produced by a diverse group of clostridia that are all placed in the single species *Clostridium botulinum* (7). The toxin acts specifically on mammalian peripheral nerves and causes a flaccid paralysis by preventing the release of acetylcholine at the neuromuscular junction (reviewed in references 24 and 25). Botulism can occur in humans when toxin is ingested in food or when *C. botulinum* infects the intestinal tracts or wounds of infants and adults (26). Infant botulism is the most common form of botulism today (e.g., in 1987 there were 86 confirmed cases of infant botulism and 20 food-borne cases [C. Hatheway, presentation of Centers for Disease Control reports at the Interagency Botulism Research Coordinating Committee Meeting, Gaithersburg, Md., October 1988]). Nearly all cases of infant botulism have been caused by proteolytic strains (group I) of toxin serotypes A and B (1, 10, 18, 26).

Little is known of the biological factors that influence growth and toxin production in foods and in the human intestine. Colonization of the infant gastric tract has been found to be inhibited by intestinal microflora from healthy infants (27). It is likely that competition for nutrients also affects colonization and may influence toxin production. Several investigators have shown that supplementation of complex media with certain nutrients, e.g., meat or casein digests, glucose, or tryptophan, increases the synthesis of toxin (4, 5, 12, 16). In complex media, however, it is difficult to accurately assess the effects of specific nutrients on the expression of toxin or the pathways of nutrient utilization (2). In the present study, we examined the influence of nutrition on protease and neurotoxin formation by proteolytic strains of serotypes A and B in a minimal defined medium (MI) (31). We found that protease and neurotoxin formation in *C. botulinum* Okra B and Hall A is strongly influenced by carbon and nitrogen nutrition.

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MATERIALS AND METHODS

Materials. Materials and methods for the anaerobic culturing of *C. botulinum* have been previously described (31). Intact casein for certain experiments was purified by the method of Rosenberg et al. (21); we also used essentially vitamin-free casein and an acid hydrolysate of casein, both from Sigma Chemical Co.

Bacterial strains and cultivation. *C. botulinum* Okra B, a stock culture from H. Sugiyama (University of Wisconsin, Madison), was used in most experiments. Its nutritional requirements have been previously described (31). We also examined the regulation of toxins and proteases in strain Hall A (ATCC 3502). Strains were purified by streaking for isolated colonies on Todd-Hewitt agar (Difco Laboratories) in an anaerobic chamber (80% N_2–10% H_2–10% CO_2). Purity was determined by microscopic examination. Cultures were grown in cooked-meat medium (Difco) and frozen at −80°C in cooked-meat medium supplemented with glycerol (10% [vol/vol]) as described previously (31).

All experiments were carried out in MI (31). Cells were grown at 37°C in anaerobic Hungate tubes with an N_2 atmosphere or in flasks or agar plates in an anaerobic glove box.

Neurotoxin and protease assays. Safety procedures for working with cultures and toxin of *C. botulinum* have been previously described (11). To estimate neurotoxin titers, we cleared culture fluid by centrifugation for 5 min in a Brinkmann microcentrifuge and injected 0.5 ml intraperitoneally into mice. The animals were observed for characteristic signs of botulism, and the time to death was recorded (14, 22). By this method 2×10^5 mouse 50% lethal doses (MLD_{50}) of type A toxin from the Hall A strain per ml leads to death in about 63 min. Although this assay is not as accurate as the quantal method (23), especially at lower toxin titers ($<10^5$/ml), it is rapid and useful for the examination of large numbers of samples and saves greatly in the numbers of mice that must be sacrificed.

Protease activity in culture fluids or in cells after disruption by sonication was determined by the azocasein method of Brock et al. (6). Reaction conditions were designed such that hydrolysis increased approximately linearly with time to a 3-h endpoint. The concentrations of acid-soluble azopeptides in the incubation mixtures were determined spectrophotometrically at 440 nm. One protease unit was defined as 1 μg of azocasein digested per h, where 320 μg of digested casein per ml equaled one optical density unit. Protease activity was also qualitatively determined by measuring zones of clearing on skim milk agarose plates (3). For analysis of intracellular protease activity, cells were disrupted by three 30-s bursts at 300 W with glass beads in a Braunsonic 2000 sonicator. Samples were kept on ice during sonication. Protease activity was measured after centrifugation by the azocasein method.

Chemical assays. Protein was measured with Coomassie G-250 dye reagent (Bio-Rad Laboratories). Ammonia was determined in the Nessler reaction with ammonia color reagent (Sigma).

Electrophoresis and immunoblotting. Methods for analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis have been previously described (11). In the present study, proteins were separated on a gel containing a linear gradient of 7.5 to 15% acrylamide stabilized by a 5 to 17.5% linear sucrose gradient. Bisacrylamide was used at 7.5% to facilitate electroelution during transblotting. Following electrophoresis at a constant current of 17 mA in the Laemmli procedure (13), proteins were transblotted overnight at a constant current of 200 mA onto 0.45-μm-pore nitrocellulose in buffer containing 0.0124 M Tris base, 0.096 M glycine, and 20% methanol (vol/vol) at pH 8.3. Toxin protein was reacted with antibodies to type B toxin and detected with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Boehringer Mannheim Biochemicals) by the method of Towbin et al. (28).

RESULTS

Control of protease formation by arginine. We tried to determine if nutritional regulation of proteases and neurotoxin occurred in *C. botulinum* by supplementing MI with various nutrients. In MI, group I *C. botulinum* (proteolytic serotypes A and B) requires high concentrations of arginine (≥3 g/liter) and phenylalanine (≥1 g/liter) for growth; sugars are not absolutely needed, although they do stimulate growth (31). Group I *C. botulinum* also requires eight additional amino acids and five additional vitamins at low levels for biosynthesis. To determine if the two nutrients required in high concentrations (arginine and phenylalanine) regulated protease production, we varied their concentrations independently in MI and tested for the synthesis of protease. Protease activities did not change significantly with various concentrations of phenylalanine (data not shown) but decreased in media containing high concentrations of arginine (Fig. 1). Protease activities were not changed by increased concentrations of tryptophan or tyrosine, which have been reported to affect toxin formation (5).

These initial experiments showed that protease activities were decreased in MI containing high concentrations of arginine. The decrease was significant; extracellular protease activity decreased up to 400-fold in media containing high levels of arginine (Fig. 1). Similar results were detected qualitatively with skim milk agar plates in assays of proteolytic activity (3). Protease activities were somewhat variable

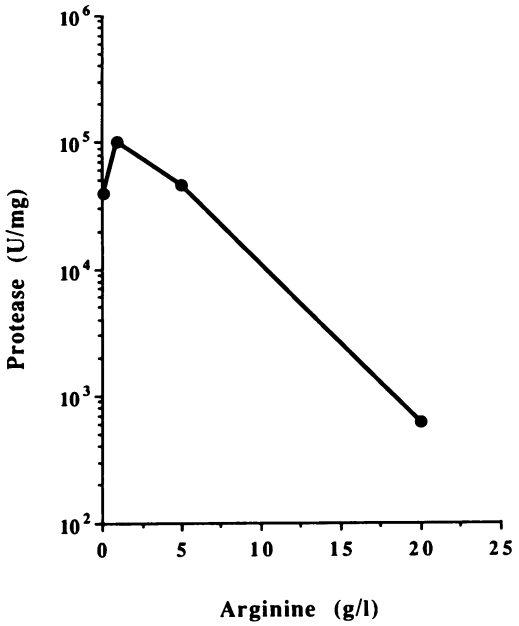

FIG. 1. Protease formation after 2 days of growth in relation to initial arginine concentrations in MI.

from experiment to experiment (up to twofold), but in many repeated trials we consistently found that high levels of arginine reduced protease activities.

The detrimental effect of arginine on protease formation seemed to be caused specifically by arginine or its catabolic products and not by artifactual changes that could be associated with arginine catabolism, such as sporulation or prevention of cell lysis. Microscopic examination of cultures in media with high and low concentrations of arginine and at various periods of growth verified that spores were not present in any of the cultures. Growth and lysis were similar in media containing various concentrations of arginine. Furthermore, decreased cell lysis was not the cause of reduced protease activities, since intracellular protease activities were also reduced in cultures grown with high concentrations of arginine (specific activities [units per milligram of protein] of intracellular protease were ≤248 and ≤88 U/mg in media containing 3 and 20 g of arginine per liter [10 g of glucose per liter], respectively). Changes in protease activities were also not caused by changes in pH. Final broth pH values increased only slightly (6.35 to 6.92) in media containing 3 to 20 g of arginine per liter. The higher pH at 20 g of arginine per liter was not due to increased liberation of ammonia from arginine, since its concentrations in media ranged from 38 to 66 μg/ml and the highest concentrations were found in media containing lower levels of arginine. It is possible that other basic molecules, such as putrefactive amines, which are readily formed by proteolytic clostridia, were responsible for the rise in pH observed with high arginine concentrations.

Protease formation followed an interesting time course (Fig. 2); proteases were synthesized during growth but progressively lost activity after growth stopped. The inactivation of proteases occurred most rapidly and to the greatest extent in cultures grown with high concentrations of arginine. Intracellular protease levels also dropped rapidly in cultures containing high concentrations of arginine, and no intracellular activity was detected after 11.5 h (data not

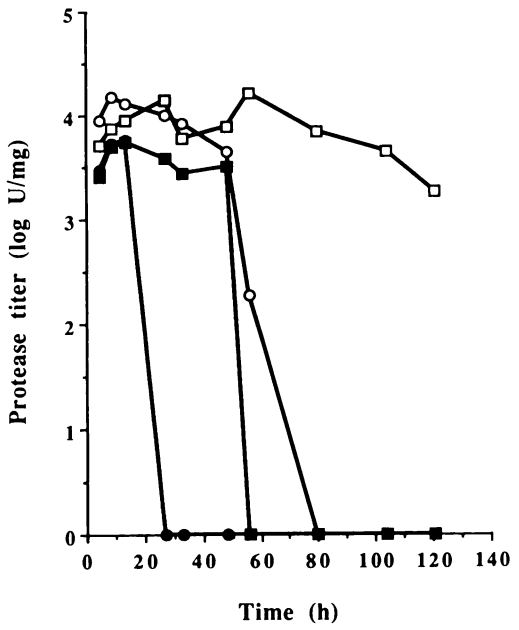

FIG. 2. Protease activities during growth in MI containing arginine and glucose at various levels (per liter): arginine, 3 g, and glucose, 10 g (○); arginine, 20 g, and glucose, 10 g (●); arginine, 3 g, and glucose, 50 g (□); and arginine, 20 g, and glucose, 50 g (■).

shown). Intracellular protease levels dropped after 57 h in media containing 3 g of arginine per liter (data not shown). Increased concentrations of glucose prevented the loss of proteolytic activity in media containing 3 g of arginine per liter and delayed the loss in media containing 20 g/liter (Fig. 2). The loss of proteolytic activity seemed irreversible because mixing broths from proteolytically active cultures or dialyzing them to remove possible loosely bound inhibitors did not restore activity. Also, broths containing inactivated protease did not inhibit trypsin or high protease activities in broths from cultures grown with low concentrations of arginine.

To further investigate protease regulation by arginine, we supplemented MI with metabolic products that might be derived from arginine, including L-citrulline, L-ornithine, L-proline, L-glutamate, L-glutamine, and NH_4Cl. We found that certain rich nitrogen sources significantly decreased protease formation relative to that in control medium with no additions (Table 1). Ammonia significantly decreased protease titers, suggesting that protease repression is not specific for arginine but instead probably is related to the availability of nitrogen in the environment.

Arginine is catabolized by the deiminase pathway in *C. botulinum* (8) and provides nitrogen, energy, and metabolic intermediates for biosynthesis. Therefore, in addition to control by nitrogen metabolism, arginine control of protease might be related to energy supply. Cultures containing 50 g of glucose per liter did not markedly change their production of protease during growth as compared with those containing 10 g of glucose per liter, but the inactivation of protease was delayed in the former (Fig. 2). The reduction in protease activity in the presence of 20 g of arginine per liter added at time zero was compared with the effect on protease activity of arginine added incrementally in four additions to a final concentration of 20 g/liter. Protease activity did not decrease when 20 g/liter was added incrementally and was comparable to the activity found with 3 g/liter.

TABLE 1. Influence of nitrogen metabolites on protease titers in MI

Supplement[a]	Protease activity (U/mg)[b]	Fold decrease
None	17,000	
Citrulline	3,200	5.3
Ornithine	6,500	2.8
Proline	5,100	3.3
Glutamate	2,900	5.9
Glutamine	6,300	2.7
NH_4Cl	2,000	4.8
Arginine	1,000	17

[a] Each supplement was provided at a concentration equivalent to that of nitrogen in 17 g of arginine per liter.
[b] Measured after 3 days of growth.

Role of casein in protease production. To further examine the nutritional regulation of protease, we tried supplementing media containing high and low levels of arginine with intact casein, which can provide nitrogen and energy through proteolysis. Casein improved the growth of *C. botulinum* (Table 2). The addition of casein counteracted the repression of proteases by arginine (Table 2) and also reduced the inactivation of proteases after growth (data not shown).

Regulation of neurotoxin by arginine. The formation of active botulinum toxin involves the synthesis of a single polypeptide protoxin and proteolytic cleavage of protoxin to the active dichain form. Since cleavage is mediated by proteolytic activity, we were interested in whether toxin formation and activation were influenced by arginine catabolism. We found that in MI containing 20 g of arginine per liter (a high level), toxin titers decreased from 50,000 MLD_{50}/ml of culture broth to approximately 50 MLD_{50}/ml (Table 3). Trypsinization did not increase the toxicity with high levels of arginine. The synthesis of neurotoxin in the Hall A strain was also regulated by arginine, as toxin titers decreased from 20,000 MLD_{50}/ml of culture broth to approximately 10 MLD_{50}/ml with high arginine supplementation (Table 3).

Toxin production followed the same metabolic patterns of regulation as those described above for protease; its forma-

TABLE 2. Influence of intact casein on protease production

Arginine level (g/liter)	Casein supplement (g/liter)	Maximum growth[a]	Protease	
			U/ml[b]	U/mg of cell
0.1	0	0.38	9.3×10^2	2.6×10^4
1.0	0	0.54	1.3×10^3	2.6×10^3
5.0	0	0.95	1.6×10^3	1.8×10^3
20.0	0	0.95	1.5×10^2	1.7×10^2
0.1	1	0.48	2.4×10^3	5.5×10^3
1.0	1	0.62	2.1×10^3	3.7×10^3
5.0	1	0.85	2.4×10^3	3.1×10^3
20.0	1	0.90	1.9×10^3	2.3×10^3
0.1	10.0	0.85	2.4×10^3	3.1×10^3
1.0	10.0	1.1	2.6×10^3	2.6×10^3
5.0	10.0	1.05	2.7×10^3	2.8×10^3
20.0	10.0	0.95	2.8×10^3	3.2×10^3

[a] Maximum A_{660} after 5 days of growth.
[b] Owing to the presence of casein, accurate protease specific activities could not be determined.

TABLE 3. Influence of arginine and casein on toxin formation in *C. botulinum* Okra B and Hall A

Strain	Supplement in medium (g/liter)	Maximum growth[a]	Neurotoxin titer (MLD_{50}/ml)
Okra B	Arginine (1)	0.78	5×10^4
	Arginine (3) (control)	0.90	1×10^4
	Arginine (5)	0.91	5×10^2
	Arginine (20)	0.84	5×10^1
	Arginine (3) + NH_4Cl (68)	0.95	1×10^2
	Arginine (3) + glucose (50)	1.0	4.5×10^3
	Arginine (20) + glucose (50)	0.95	1×10^4
	Arginine (0.1) + casein (10)	0.68	3×10^4
	Arginine (1) + casein (10)	0.83	6×10^4
	Arginine (5) + casein (10)	0.98	1×10^3
	Arginine (20) + casein (10)	0.98	7×10^2
Hall A	Arginine (3)	0.85	2×10^4
	Arginine (20)	0.90	<10

[a] A_{660}.

tion was impaired by arginine and central nitrogen metabolites. For example, the addition of high levels of NH_4Cl caused a reduction in toxin titers from 10,000 MLD_{50}/ml (control) to 100 MLD_{50}/ml. Also, glucose reduced the repressive effects of arginine. When glucose was present at 50 g/liter, increasing the level of arginine from 3 to 20 g/liter did not result in a decrease in toxin titers but rather an increase. We also found that toxin titers increased considerably in MI containing casein or enzymatic casein hydrolysates but that casein did not override arginine repression of toxin as strongly as it did protease production (Table 3).

Neurotoxin titers assayed with mice and protein immunoblots throughout the growth cycle indicated a correlation between toxin formation, immunoreactive proteins, proteolytic activity, and protoxin activation (Fig. 3). The culture supernatant from media containing high levels of arginine (20 g/liter) (lanes 1, 4, 5, 8, and 9) all showed decreased concentrations of polypeptides immunoreactive with antibodies raised against type B neurotoxin. In contrast, the highest concentrations of toxin proteins were observed in media containing low levels of arginine (1 g/liter) (lanes 2, 3, 6, 7, 12, and 13). Also, considerably more fragmentation of proteins that reacted with toxin antibodies on immunoblots was observed for the cultures producing more protease (Fig. 3). These results indicate that the synthesis, activation and, possibly, degradation of toxin were all controlled by the nutritive composition of the growth medium, particularly by the presence of arginine.

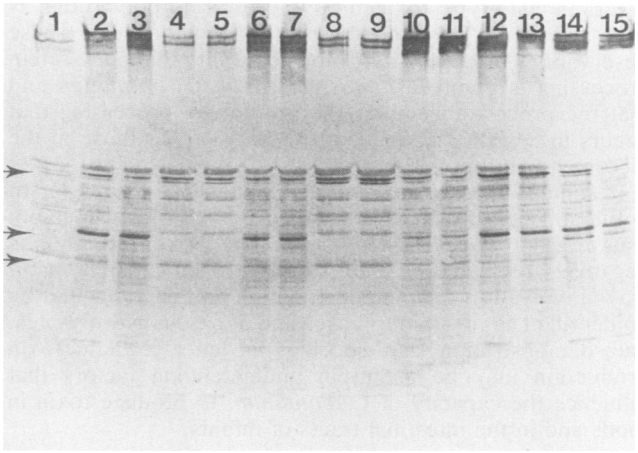

FIG. 3. Immunoblot analysis of neurotoxin in culture supernatant from MI containing low and high levels of arginine and supplements. All samples were taken after 5 days of growth, and 5 μg of protein was loaded in each lane. Lanes: 1, 20 g of arginine plus 5 g of Casamino Acids (rich N source per liter); 2 and 3 (duplicate samples), 1 g of arginine plus 5 g of Casamino Acids per liter; 4 and 5, 20 g of arginine plus 50 g of glucose (excess glucose) per liter; 6 and 7, 1 g of arginine plus 50 g of glucose per liter; 8 and 9, 20 g of arginine per liter; 10 and 11, 5 g of arginine per liter; 12 and 13, 1 g of arginine per liter; 14 and 15, 0.1 g of arginine per liter. The arrows indicate protoxin (150,000 daltons) and dichain toxin subunits of 100,000 and 50,000 daltons.

DISCUSSION

Many investigators who study *C. botulinum* have been frustrated by the tendency of the organism to sporadically produce low toxin titers in different batches of the same medium and also by its tendency to progressively produce decreasing quantities of toxin on successive subcultures. The sporadic formation of high toxin titers from batch to batch and variations in different media suggest that toxin formation is regulated by nutritional and environmental factors. We have shown in this study that the availability of nitrogen and energy sources, particularly the availability of arginine, influences toxin production by *C. botulinum*. Our data also suggest that factors in intact casein or enzymatic digests stimulate toxin formation or decrease arginine repression.

Problems with the consistent production of high titers of tetanus toxin have also been described (15). Early studies were directed to understanding the nutritional factors involved in tetanus toxin formation (9, 20). These workers found that good yields of tetanus toxin were not obtained unless a small quantity of heart infusion was added to the production medium. They recognized the value of using a defined medium for the identification of stimulatory and inhibitory factors. "If it were possible to grow the tetanus organism on a medium containing only chemically defined substances of low molecular weight, it should become a relatively straightforward matter to study and control the factors involved in toxin formation, and to obtain a uniform product free from any possible antigenic material other than the specific substance desired," they stated (19). They showed that the stimulatory factor was a peptide possessing histidine (20). In addition to the observation that histidine peptides stimulated tetanus toxin production, other investigators showed that supplementation of media with certain amino acids, including serine, glutamate, aspartate, glutamine, asparagine, and free histidine, reduced toxin yields (29). As with *C. tetani*, therefore, it appears that botulinum toxin formation and proteolytic activation are controlled by inhibitory and stimulatory factors related to nitrogen metabolism. When negative regulation and positive regulation are involved, as is true with many catabolic pathways (17), it is often difficult to identify nutrients involved in control, since opposing interactions may take place. The use of minimal media is usually required in such studies and was essential for our identification of arginine as an important nutrient in protease and toxin formation.

We have obtained evidence in this study that posttranslational processing of toxin and inactivation of proteases are regulated by arginine. Nontoxigenic mutants that accumulate a very large polyprotein have been isolated in our laboratory (B. Micales and E. A. Johnson, unpublished

data). In wild-type toxigenic cultures, this polyprotein is only transiently detected during the growth cycle. These observations raise the interesting possibility that polyprotein processing is involved in toxigenesis in *C. botulinum* and that the process resembles the proteolytic processing that occurs in certain mammalian viruses, such as those in the picornavirus group (30).

In summary, we have demonstrated that the expression of botulinum neurotoxin is controlled by nutritional metabolism in group I strains of *C. botulinum*. Toxin formation is negatively regulated by energy and nitrogen catabolic pathways involved in arginine degradation and is stimulated by unidentified factors in purified casein or casein hydrolysates. Our demonstration that specific nutrients regulate toxin production may be useful in understanding factors that influence the capacity of *C. botulinum* to produce toxin in foods and in the intestinal tracts of infants.

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