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Oxidation of Nicotinic Acid by a *Bacillus* Species: Regulation of Nicotinic Acid and 6-Hydroxynicotinic Acid Hydroxylases

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The first two enzymes employed by a *Bacillus* species for the dissimilation of nicotinic acid are coordinately induced. The inducer of the enzymes appears to be 6-hydroxynicotinic acid, the product of the first enzyme in the pathways. Synthesis of the enzymes is repressed by glucose when ammonium is present in the medium, but not when nicotinic acid is the sole nitrogen source. The possible significance of the coordinate induction and unusual repression is discussed.

Nicotinic acid (NA) serves as a sole source of carbon and energy for a *Bacillus* species isolated from soil (6). The first two reactions in the degradation pathway are hydroxylation of NA to 6-hydroxynicotinic acid (6-HNA) followed by a second hydroxylation which yields 2,6-dihydroxynicotinic acid (2,6-DHNA). The NA and 6-HNA hydroxylases have been purified and characterized as large iron-containing flavoproteins (10). Both enzymes use water as the source of oxygen atoms for the hydroxylations (11).

Previous work showed that induction of the NA degradation pathway by NA in glucose-grown resting cells was very slow, occurring only after a 6- to 10-hr lag period (6). This suggested the possibility that some intermediate other than NA might be the inducer of the pathway. We report here that NA hydroxylase and 6-HNA hydroxylase are coordinately induced by the product of the first reaction, 6-HNA. We also report the results of studies of regulation of the hydroxylases by catabolite repression.

MATERIALS AND METHODS

Organism and culture conditions. The *Bacillus* species, originally described by Ensign and Rittenberg (6), was grown in a medium containing (per liter of distilled water) 13.3 g of K_2HPO_4, 4.0 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4$, 5.0 ml of trace salts solution, and 10.0 ml of vitamins solution. The trace salts solution contained: 2.0 g of $CaCl_2 \cdot 2H_2O$, 1.0 g of $MnSO_4$, 0.5 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $CoCl_2 \cdot 5H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.01 g of $ZnSO_4 \cdot 7H_2O$, and 0.01 g of $NaMoO_4 \cdot 2H_2O$, in 1 liter of 0.1 N HCl. Vitamins were added from a filter-sterilized stock solution to give final concentrations of 4 μg of thiamine-hydrochloride/ml, 0.8 μg of biotin/ml, and 0.04 μg of vitamin B12/ml. All three were required for growth. The medium, complete except for a carbon source, is referred to as M medium. Carbon sources were added from concentrated solutions, sterilized separately.

Cultures were incubated at 30 C on rotary shakers or with forced aeration through sintered-glass spargers. Growth was measured with a Klett-Summerson colorimeter at 540 nm (green filter).

Sampling procedures and preparation of extracts. Time course experiments were conducted in 6-liter carboys containing 3 to 4 liters of medium. Samples of about 200 ml were removed and poured over flake ice made from distilled water. Chloramphenicol was added at a final concentration of 50 μg/ml. The cells were collected by centrifugation at 5,000 × *g* for 5 min at 4 C, washed once in cold 0.02 M phosphate buffer, *p*H 7.4, containing 50 μg of chloramphenicol/ml, and resuspended in 2 ml of the same buffer. Cell-free extracts were prepared by forcing the cell suspension through a prechilled French pressure cell and then centrifuging the disrupted cell preparation at 20,000 × *g* for 1 hr at 4 C. The supernatant fluid was removed and assayed for the hydroxylating activities. In other experiments, cells were collected by centrifugation, washed, suspended in cold buffer without chloramphenicol, and made into extracts by the procedure described above. The extracts were either assayed immediately or stored at −15 C. In either case, all of the samples for a given experiment were assayed on the same day.

Analytical procedures. The enzymatic conversion of NA to 6-HNA was assayed by following the increase in absorbance at 295 nm, a maximum of

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absorbance characteristic for 6-HNA. Assays were performed in a Cary 15 recording spectrophotometer (Applied Physics Corp.). Reaction mixtures contained 30 μmoles of potassium phosphate buffer, pH 7.4, 0.1 μmole of methylene blue, 0.5 μmole of NA, and enzyme in a 3.0-ml total volume. The conversion of 6-HNA to 2,6-DHNA was assayed at 322 nm, a maximum of absorbance characteristic for 2,6-DHNA, in a similar reaction mixture which contained 6-HNA instead of NA. A unit of activity is defined as the conversion of 1 nmole of substrate to product in 1 min at room temperature (about 25 C). Specific activities are expressed as units per milligram of protein.

Protein was determined by the Folin phenol method (15), using lysozyme as a standard. The glucose content of growth liquor was determined with glucose oxidase (Glucostat reagent, Worthington Biochemical Corp.) after the cells were removed by filtration through membrane filters. The ammonium nitrogen content of these filtrates was determined with Nessler's reagent (23).

Determination of $^{14}CO_2$. The release of $^{14}CO_2$ from carboxy-labeled NA was measured radiorespirometrically by the procedure of Krulwich and Ensign (13). A 0.5-ml sample of washed cells was inoculated into 5.0 ml of M medium. The carbon sources to be tested and 0.5 μCi of NA-7-^{14}C (specific activity 5.03 mCi/mmole, New England Nuclear Corp.) were added. Sterile air was bubbled through the reaction mixture which was immersed in a 30 C water bath. The $^{14}CO_2$ was collected in phenethylamine and was counted in a Packard Tri-Carb scintillation spectrometer using Bray's scintillation mixture (3).

Chemicals. NA, maleamic acid, 6-HNA, and 2-chloronicotinic acid were purchased from Aldrich Chemical Co. The fluoro derivatives of NA were a gift of the Eli Lilly Co., Indianapolis, Ind. *N*-formylmaleamic acid was a gift of S. C. Rittenberg. The procedure of Ensign and Rittenberg was used for the enzymatic synthesis of 2,6-DHNA (6). For the synthesis of 2-hydroxynicotinic acid, the procedure of Phillips was followed (18).

RESULTS

Induction studies. The basal levels of the NA and 6-HNA hydroxylases in uninduced cells were determined prior to studying regulation of the enzymes. Cells grown for at least 10 generations on M medium with glucose, glycerol, or succinate as carbon source were harvested in the late exponential phase of growth. Cell extracts were prepared from duplicate cultures grown on each of the three substrates. Each extract was assayed for NA and 6-HNA hydroxylase activity. No activity was detected in any of the extracts, even when the assay periods were extended to several hours and the reaction mixtures contained as much as 8 mg of protein/ml. These assays are sensitive enough to detect 0.1% of the fully induced levels of the two hydroxylases. The levels of these enzymes in uninduced cells are therefore less than this.

Experiments were designed to compare the effectiveness of NA and 6-HNA as inducers of the respective hydroxylases. NA (0.1% final concentration) was added to a culture growing exponentially on succinate. The same amount of 6-HNA was added to another culture. At various times, samples were removed, extracts of the cells were prepared, and the levels of NA and 6-HNA activity were determined. The pattern of induction of the hydroxylases by the two substrates is illustrated in Fig. 1. When NA was added (Fig. 1A), no activity was detected until between 30 and 60 min. The specific activities of both enzymes then increased at the same rate for 5 hr. The lag period before appearance of the hydroxylase activities was shorter when 6-HNA was added to the cells (Fig. 1B). Detectable levels of both hydroxylases were evident at 30 min. The subsequent rate of increase in the specific activities of both enzymes was nearly the same as was obtained with NA as inducer. The results of this experiment suggest that NA is not the inducer of the two hydroxylating enzymes and suggest, instead, that 6-HNA is the inducer.

Known intermediates in the pathway of NA degradation after 6-HNA are 2,6-DHNA, maleamic acid, and maleic acid (6). The pathway then merges with the tricarboxylic acid cycle at the level of fumarate. It is possible that *N*-

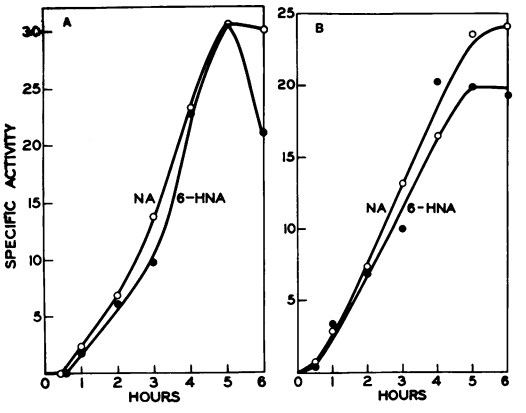

FIG. 1. *Time course for induction of the nicotinic acid (○) and 6-hydroxynicotinic acid (●) hydroxylases by nicotinic acid (A) or 6-hydroxynicotinic acid (B). At zero hour, inducer (final concentration 0.1%) was added to a culture which was growing exponentially on succinate. At the indicated times, cells were removed from the cultures, cell-free extracts were prepared, and the specific activities of the hydroxylases (nmole of product formed per minute per milligram of protein) in the extracts were determined.*

formylmaleamic acid is an intermediate between 2,6-DHNA and maleamic acid. Behrman and Stanier (2) suggested that *N*-formylmaleamate is the product of ring cleavage during the degradation of NA by *Pseudomonas putida*. Gauthier and Rittenberg (8), studying the same bacterium, proved conclusively that the product of the pyridine-ring cleavage of nicotine is maleamate and not the *N*-formyl derivative. Each of the known pathway intermediates and *N*-formylmaleamic acid were tested for the ability to induce synthesis of the NA and 6-HNA hydroxylase activities. Maleamic acid, maleic acid, or *N*-formylmaleamic acid at a final concentration of 0.1% or 2,6-DHNA at 0.05% was added to a culture growing on succinate. The cultures were incubated for 4 hr (2,6-DHNA) or 12 hr (other substrates). The shorter incubation time for 2,6-DHNA was necessary because of its tendency to spontaneously oxidize to a blue pigment (6). Extracts of the cells were prepared and assayed for NA and 6-HNA hydroxylase activity. Maleamic acid, maleic acid, and 2,6-DHNA do not induce hydroxylase synthesis. NA-grown resting cells readily metabolize each of these compounds. Thus, although the compounds permeate the cells, they do not induce synthesis of the permease.

These experiments lead to the conclusion that 6-HNA induces the two hydroxylases in a coordinate manner. The slower induction by NA would be explained by its conversion to 6-HNA by NA hydroxylase which, although undetectable in the uninduced cells, is presumably present at very low levels.

The specific activities of coordinately controlled enzymes vary proportionally at different levels of induction so that when the various specific activities are plotted against one another, a straight line results (1). A plot of this type for the two hydroxylases is shown in Fig. 2. The values were taken from the time course experiments shown in Fig. 1 and from an experiment where the specific activities of the two hydroxylases were determined in extracts of cells grown with concentrations of NA and 6-HNA from 5×10^{-4} to 2×10^{-2} M. A straight line plot was obtained, indicating that the hydroxylases are coordinately controlled.

Various pyridine- and benzene-ring compounds were tested for their ability to induce the two hydroxylases. For screening purposes, a qualitative test of induction was used. Cultures were grown on M medium containing 0.1% succinate plus 0.1% of the test compound. A negative control containing only succinate and a positive control containing succinate plus NA were included. After growth for two to three generations, the cells were sedimented by centrifugation and suspended in 5.0 ml of 0.02 M phosphate buffer, *p*H 7.4, containing 0.1% neotetrazolium chloride and 2 mM NA. The dye accepts protons which are produced during NA hydroxylation; the result is precipitation of a red formazan. Formation of the precipitate indicates that the hydroxylases were induced. The results obtained are summarized in Table 1. Of the 30 compounds tested, 7 appeared to induce synthesis of the hydroxylases. This was confirmed by assaying hydroxylase activity in extracts of the cells grown on each of the seven effective compounds.

NA-grown resting cells were tested for their ability to oxidize each of the seven inducing compounds. NA, 6-HNA, 2-hydroxynicotinic acid, and nicotinamide were oxidized completely to the level of CO_2, NH_3, and water. The cells partially oxidized 2-amino and 5-fluoronicotinic acids with an uptake of 0.5 and 1.0 μmoles of O_2 per μmole of substrate, respectively. The cells did not oxidize 6-floronicotinate; it is apparently a gratuitous inducer of the hydroxylases.

Repression studies. When succinate-grown cells were inoculated into M medium containing 0.1% NA plus 0.1% succinate, rapid growth occurred with no subsequent diauxic lag. Growth was faster (2.8-hr doubling time) than with either carbon source alone (4.3-hr doubling time). In another experiment, two culture tubes containing 0.2% NA plus 10 μCi of ^{14}C-NA, one with and one without 0.05% succinate, were inoculated with succinate-grown cells. The time course of induction, measured by the release of $^{14}CO_2$ from NA, was identical in the two cultures. These results show that succinate does not repress synthesis of the NA pathway enzymes.

Similar experiments showed that glucose is an effective catabolite repressor of the pathway. Diauxic growth occurred when glucose-grown cells were inoculated into M medium containing 0.2% NA and 0.1% glucose (Fig. 3). Exponential growth ceased after 5 hr. At this time, glucose had been depleted, and no detectable hydroxylase activity was present in the cells. After a 1.5- to 2-hr lag, both NA and 6-HNA hydroxylase activities appeared. About 1 hr later the cells began to divide at the slower rate characteristic of growth on NA.

NA can be used by this organism as both a carbon and nitrogen source when ammonium sulfate is omitted from the growth medium. It may be that in nature the organism might fre-

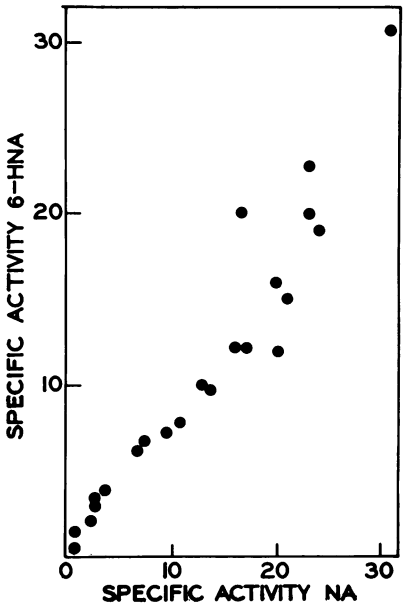

FIG. 2. *Coordinate induction of nicotinic acid hydroxylase (NA) and 6-hydroxynicotinic acid hydroxylase (6-HNA). Values are taken from the time-course experiment (see Fig. 1) and from an experiment where nicotinic acid or 6-hydroxynicotinic acid was used as inducer at concentrations from* 5×10^{-4} *to* 2×10^{-2} M.

TABLE 1. *Survey of ability of various compounds to induce synthesis of nicotinic acid and 6-hydroxynicotinic acid hydroxylases*

Induce	Do not induce
Nicotinic acid	2,4-Dicarboxylpyridine
6-Hydroxynicotinic acid	3-Methylpyridine
2-Hydroxynicotinic acid	3-Acetylpyridine
Nicotinamide	*m*-Hydroxybenzoic acid
2-Aminonicotinic acid	2-Hydroxypyridine
5-Fluoronicotinic acid	Quinolinic acid (2,3-dicarboxylpyridine)
6-Fluoronicotinic acid	8-Hydroxyquinoline
	3-Hydroxypyridine
	Catechol
	p-Hydroxybenzoic acid
	Protocatechuic acid
	2-Hydroxy-6-methyl nicotinic acid
	4-Hydroxypyridine
	Pyridine-3-sulfonic acid
	Isoniazid
	m-Hydroxybenzoic acid
	Mandelic acid
	Benzoic acid
	Pyridine
	Isonicotinic acid (4-carboxylpyridine)
	Picolinic acid (2-carboxylpyridine)
	Nicotine
	Dipicolinic acid (2,6-dicarboxylpyridine)

quently be faced with conditions where lack of available nitrogen limits its growth. In such a case, it would be advantageous to be able to degrade NA to obtain its nitrogen atom regardless of the presence of a repressing carbon source. This was tested by inoculating washed glucose-grown cells into M medium containing 0.2% glucose and 0.1% NA but only 0.015% $(NH_4)_2SO_4$. Diauxic growth resulted (Fig. 4). The first phase of growth terminated when the ammonium nitrogen was depleted from the medium. Approximately half of the glucose had been used. During the 1.5-hr lag period, the NA and 6-HNA hydroxylases were synthesized. The cells then began to grow, presumably using the pyridine-ring nitrogen. Derepression occurred even though the residual glucose in the medium (about 0.1%) was sufficient to repress enzyme synthesis when nitrogen was not limiting (see Fig. 3). These results show that repression of the hydroxylases by glucose is relieved when NA is the only nitrogen source.

DISCUSSION

The results of this study show that the first two enzymes, NA and 6-HNA hydroxylase, involved in NA dissimilation by a *Bacillus* species are induced coordinately. The inducer of the two enzymes appears to be 6-HNA, the product of the first hydroxylase. Coordinate induction of enzymes is a common occurrence when bacteria degrade benzene-ring compounds. For a complete discussion of this topic, see the recent reviews by Dagley (4) and by Hegeman and Rosenberg (9) and the recent papers by Rosenberg (20) and by Johnson and Stanier (12). The product of the first reaction involved in the catabolism of a substrate by bacteria is often the inducer of enzymes for the entire pathway (7, 14, 17, 21).

Coordinate induction of blocks of dissimilatory enzymes simplifies matters for a bacterium at the genome level. Significantly fewer genes are required for regulation of synthesis of a block of enzymes than when each enzyme is regulated independently. Coordinate induction of the first two enzymes involved in NA dissimilation by 6-HNA may be of importance to the organism used in this study for another reason. In nature, the bacterium would only rarely encounter a sufficient amount of NA to be utilized for carbon and energy. Most of the

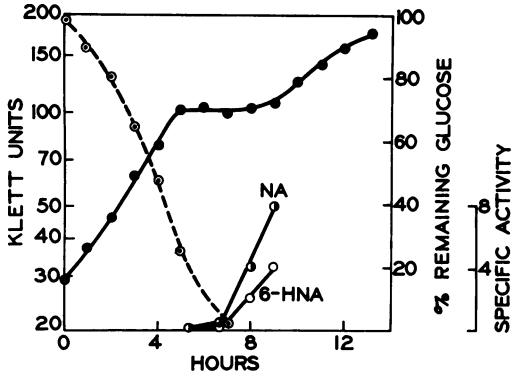

FIG. 3. *Repression of the nicotinic acid and 6-hydroxynicotinic acid hydroxylases by glucose. Glucose-grown cells were inoculated into M medium containing 0.1% glucose and 0.2% nicotinic acid. At the times indicated, growth (●), glucose remaining in the medium (⊙), and the specific activities of nicotinic acid hydroxylase (◑) and 6-hydroxynicotinic acid hydroxylase (○) in cell-free extracts were determined.*

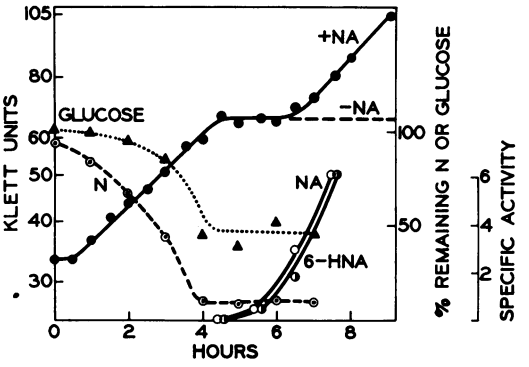

FIG. 4. *Derepression of the nicotinic acid and 6-hydroxynicotinic acid hydroxylases in the presence of glucose when nitrogen is limiting. Glucose-grown cells were inoculated into M medium containing 0.2% glucose and 0.1% nicotinic acid, with ammonium sulfate reduced to 0.015%. At the times indicated, growth (●), glucose remaining in the medium (▲), ammonia nitrogen remaining in the medium (⊙), and the specific activities of nicotinic acid hydroxylase (○) and 6-hydroxynicotinic acid hydroxylase (◑) in cell-free extracts were measured.*

time it would be necessary to synthesize NA for incorporation into pyridine-ring-containing coenzymes. It would be disadvantageous for the bacterium to synthesize a compound which might in turn induce the enzymes for its own degradation. The *Bacillus* seems to have solved this potential dilemma by maintaining extremely low uninduced levels of NA hydroxylase and by using the second intermediate in the pathway as the inducer. In this way, the organism can still take advantage of large concentrations of NA, if ever encountered, by a slow induction of the catabolic enzymes. At the same time, the danger of induction by intracellular molecules of NA is minimized when the organism is growing on other substrates.

The induction of NA and 6-HNA hydroxylases is fairly specific. Only pyridine-ring compounds carboxylated in the 3 position will induce. Also, with the exception of 6-fluoronicotinic acid, the number 6 position must be free or be substituted with a hydroxyl group. The closest structural analogues to NA and 6-HNA in the benzene series, benzoic acid and *p*-hydroxybenzoic acid, do not induce. These observations support the conclusion derived from time course induction studies that 6-HNA is the true inducer of the hydroxylases.

Catabolite repression of enzymes involved in aromatic ring degradation is a general phenomenon (16, 22). Catabolite repression of the NA and 6-HNA hydroxylases by glucose requires the presence of ammonia nitrogen; derepression occurs when NA is the sole nitrogen source. This pattern of catabolite repression might be of survival value to the organism. Given a balanced supply of usable carbon and nitrogen, the enzymes for NA breakdown would not be synthesized. If faced with nitrogen starvation when plentiful carbon sources exist, the organism could adapt to use any NA which might be available as a nitrogen source. Synthesis of enzymes for degradation of amino acids by bacteria are also derepressed when the amino acid is the sole nitrogen source (14, 19). Decker and Bleeg (5) found that the first two enzymes for nicotine oxidation by *Arthrobacter oxydans* are coordinately controlled, and synthesis of the enzymes is repressed by glucose and ammonia but not by either alone.

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