Hyperproduction and Purification of Nicotinamide Deamidase, a Microconstitutive Enzyme of Escherichia coli

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SUMMARY

Nicotinamide deamidase is synthesized constitutively in very small amounts by Escherichia coli. Mutants characterized by up to 50-fold higher deamidase activity have been isolated by mutagenesis and selection on minimal plates containing nicotinamide as the sole nitrogen source. These mutants still synthesize the enzyme constitutively.

The enzyme was purified 200-fold. The final specific activity from one mutant is 10,000 times greater than the specific activity in the wild type. The molecular weight of the enzyme is about 30,000. Kinetic constants and heat stabilities of purified enzymes from two mutant strains and the wild type are identical. Gel electrophoresis has further suggested that the mutations result in over production of unaltered nicotinamide deamidase. Various hyperconstitutive mutations and the deamidase structural gene are cotransduced at high frequency, and thus lie close together.

The activities of certain bacterial enzymes are very low and cannot be increased by changing the growth conditions. Both the practical problem of producing these enzymes in sufficient amounts and the regulation of their synthesis merit study. An enzyme is commonly coded for by a single gene, and yet different enzymes are made constitutively in different amounts. Thus, some genes must be more active than others. Induction-repression mechanisms provide control in some cases. But, as we have proposed earlier, a regulatory mechanism that holds enzyme production to a low level without requiring repressors might be energetically more efficient for the minor enzymes (1). A few mutations that increase production of constitutive proteins have been noted: dihydrofolate reductase of Diplococcus pneumoniae (2), Escherichia coli lac repressor (3), and glucose 6-phosphate dehydrogenase of E. coli (4).

Nicotinamide deamidase production by E. coli is a good system for study of this problem. The activity of the enzyme is about 3 nmoles of nicotinamide hydrolyzed per min per mg of protein (5, 6), a value several orders of magnitude lower than the activity of many enzymes involved in major bacterial pathways. Nicotinamide deamidase is active in the cyclical salvage pathway for production of DPN from nicotinamide (7, 8); it catalyzes the hydrolysis of nicotinamide to nicotinic acid.

EXPERIMENTAL PROCEDURE

Bacterial Strains—E. coli K12 -2000α was used as a wild type strain for nicotinamide deamidase. It is a lac+ recombinant of E. coli 2320 and CS101. E. coli K12 nadD6 isolated by Dr. C. Yanofsky is a nicotinic acid auxotroph (5). A double mutant isolated by Dr. T. K. Sundaram (9) is E. coli W3880-nam11, a nicotinic acid auxotroph (nadB30), and is also deamidase negative (pncA). Both mutants were supplied by Dr. R. K. Gholson whose nomenclature we follow (10).

Bacterial Culture—Strains were stored at 4° on Difco nutrient agar slants and routinely subcultured every 3rd month. Growth was at 37° with aeration by swirling in nutrient broth plus 5 mg per liter of thiamine·HCl and was followed by turbidity. Minimal medium (M63) contained per liter: 13.6 g of KH2PO4, 0.2 g of MgSO4·7H2O, 2 g of (NH4)2SO4, 0.5 mg of FeSO4·7H2O, and 5 mg of thiamine·HCl, neutralized to pH 7.0 with KOH. Glucose, autoclaved separately, was added at 4 g per liter of final concentration. Nicotinamide, nicotinic acid, and their derivatives were sterilized by passing their solutions through sterile Millipore HA filters. Nicotinamide was added at 0.1 mM as a vitamin or 30 mM as a nitrogen source.

Reagents—[carboxyl-14C]Nicotinamide (13 mCi per mmole) was obtained from Nuclear Radio-Chicago, Inc., and Amersham-Searle, nicotinic acid from Mann, protamine sulfate and NAD from Sigma, N-methyl-N'-nitro-N'-nitrosoguanidine and nicotinamide from Aldrich Chemical Company, and penicillin G and NADP* from Calbiochem.

Assay—Nicotinamide deamidase was assayed by the conversion of [14C]nicotinamide to nicotinic acid. The 0.5 ml of standard reaction mixture contained 0.1 ml of 0.5 mM potassium phosphate buffer, pH 7.0; 0.3 ml of 1.4 mM nicotinamide containing 2 μCi per ml of 14C and 0.1 ml of enzyme preparation at a concentration chosen to yield less than 60% conversion. The ATP and MgSO4 previously added (3) were found to be
unnecessary. The reaction mixture was incubated with gentle swirling at 37° for 15 min. Reactions were halted by placing the tubes into a boiling water bath for 2 min. Then 0.25 ml of 0.1 M nicotinamide plus 0.1 M nicotinic acid were added as indicators for chromatography. Ten microliters of each mixture were applied to Eastman silica gel TLC plates (20 x 20 cm) (type K301R). The chromatograms were developed using n-butyl alcohol-10% ammonium hydroxide (19:1), in which nicotinamide has an RF of 0.65 and nicotinic acid an RF of 0.35 (11). The small dense spots were identified under ultraviolet light, marked, cut out, placed in Bray's solution, and counted for radioactivity. The counts were corrected for background by subtracting the counts given by a sample containing no enzyme. Activity of a crude extract of wild type cells was proportional to incubation time up to at least 40% conversion. Nicotinamide deamidase activity was calculated from the percentage of conversion; 1 unit of enzyme produces 1 nmole of nicotinic acid per min. Protein was determined using the Folin method of Lowry et al. (21).

Mutant Selection—E. coli 2000α was treated with N-methyl-N'-nitro-N-nitrosoguanidine (13), grown overnight in nutrient broth, washed, and plated on minimal agar which contained 30 μM nicotinamide as the sole nitrogen source. Colonies that appeared after 2 days were tested for their hyperconstitutive production of the enzyme. We have named the hyperconstitutive locus pncH. Of six mutant cultures selected, one, pncH6, was again mutagenized and selected in order to get another set of mutants.

Transduction—E. coli W3889-nam11, the pncA, nadB double mutant, was transduced with λ phage grown on the various pncH mutants according to the procedure of Yanofsky and Lennox (14). Transductants were plated on three different media. If the phage introduces either pncA or nadB a transductant can grow on minimal agar with 0.1 mM nicotinamide as a vitamin source. One must subtract the nadB transductants, which grow on minimal agar, from the total in order to determine the pncA transductants. Transductants that received both the pncA and pncH genes (cotransductants) were scored after 1 to 2 weeks on minimal plates that contained 30 mM nicotinamide as the sole nitrogen source.

RESULTS

Constitutive Production of Nicotinamide Deamidase

As Imande has reported (5), nicotinamide deamidase is produced at the same level under several growth conditions. It was found at very similar activities in exponential and resting cells. Addition of nicotinic acid derivatives did not change enzyme production (Table I). These compounds were supplied in the concentration ranges required for optimal growth of a nicotinate-requiring mutant, E. coli nadB6 (0.3 μM nicotinamide or 2 μM nicotinate). We noted, however, that this mutant had only one-third the deamidase activity of the wild type.

Mutant Isolation

A series of six pncH mutants with elevated rates of deamidase production were selected following mutagenesis of E. coli 2000α as described under "Experimental Procedure." They grew more rapidly on plates containing nicotinamide as a sole nitrogen source. These mutants possessed up to 20 times the wild type enzyme level. Remutagenization of pncH6 and selection yielded six other mutants, one of which had 50 times the wild type level of enzyme (Table II). Thus, mutation could greatly increase the activity per cell of this low level constitutive enzyme.

Mixing Experiments

Activity of a 30-fold purified extract from pncH9 was not altered by addition of an equal quantity of an extract from the wild type (of much lower activity). Thus, the wild type extract does not contain an inhibitor of the enzyme.

Purification

A summary of the enzyme purification from pncH9 is given in Table III. A single colony of E. coli pncH9 inoculated into 4 liters of nutrient broth was grown to the late log phase. The
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Protamine sulfate. 110
Sonicate. 120
using 0.4 µg of protein per assay.

chilled 0.05 M NaCl plus 0.5% KCl and were frozen with acetone-Dry Ice. These could be stored in the frozen condition. Further purification was at 0-5°C unless otherwise noted.

Protamine sulfate were added dropwise with continuous stirring for 20 min, the supernatant was decanted and immediately readjusted to pH 7.0 by dropwise addition of chilled 1 N KOH.

3. Ammonium Sulfate Fractionation—The protamine sulfate supernatant was adjusted to 25 mg per ml of protein using a Diaflo model 52 apparatus with an Amicon UM-10 membrane. A saturated ammonium sulfate solution at 0°C was added dropwise with stirring to give 50% saturation. Stirring was continued for 15 min; the preparation was then centrifuged at 20,000 × g for 30 min. The supernatant was carefully decanted and ammonium sulfate was added to 50% saturation, stirred, and centrifuged as above. This precipitate was dissolved in a minimal volume of 0.05 M phosphate buffer, pH 7.0, and dialyzed against a 200-fold excess of the same buffer for 24 hours, with buffer changes approximately every 6 hours.

4. Acetone Fractionation—To the dialyzed material 3 volumes of acetone at 0°C were added dropwise, stirred for 15 min, and centrifuged at 22,000 × g for 30 min at -20°C. The supernatant was decanted and 5 additional volumes of acetone were added, as before. The precipitate was dissolved in a minimal volume of 0.05 M phosphate buffer, pH 7.0, and allowed to stand uncovered at 0°C overnight to allow acetone to evaporate.

5. Sephadex G-100 Fractionation—The dissolved material was concentrated, if necessary, to approximately 1.5 ml using a Diaflo model 12 apparatus with a UM-10 membrane. Three drops each of concentrated blue dextran and riboflavin solutions were added as markers. The sample was fractionated on a Sephadex G-100 column (14 mm x 160 cm) that had been equilibrated with 0.05 M phosphate buffer, pH 7.0. The sample was eluted with the same buffer, and 10-ml fractions were assayed for protein and enzyme. Total protein decreased throughout the elution, and enzyme appeared as a peak at about 0.35 column volume.

Properties of Enzyme

The enzyme lost little activity in several days at 0°C in phosphate buffer at pH 7.0. It had a half-life of about 2 hours at 37°C, but it was stable in the presence of 5 µM nicotinamide. Its stability was reduced in low salt concentrations. Activity of a partly purified preparation which had been stored at 0°C was increased slightly by prior incubation for about an hour at room temperature.

Kinetics

Production of nicotinic acid was proportional to added protein. Activity was approximately constant for 20 min or until about 150 nmoles of nicotinate were produced (Fig. 1). Fig. 2 shows a double reciprocal plot of velocity versus substrate concentration. The partially purified enzymes from the wild type and from two mutants pncH9 and pncH2 are compared. The K₉ values were virtually identical at about 7 × 10⁻⁵ M, although the specific activities (V₉ₜₜ) varied over a 50-fold range.

The pH optimum was approximately 7.2, as measured in 0.1 M phosphate buffer at pH 7.0. It had a half-life of about 2 hours at 37°C, but it was stable in the presence of 5 µM nicotinamide. Its stability was reduced in low salt concentrations. Activity of a partly purified preparation which had been stored at 0°C was increased slightly by prior incubation for about an hour at room temperature.

Molecular Weight Estimation

The elution volume on Sephadex G-100 of pncH9 deamidase gave, by interpolation between cytochrome c and hemoglobin, a molecular weight of approximately 30,000 (16).

Heat Stability

The partially purified enzymes from the wild type and the two mutants showed identical inactivation kinetics, with half of the
activity being lost in 5 min at 50°. When the duration of incubation was varied at 55°, the inactivation curves for the three preparations appeared to be identical (Fig. 3), again suggesting that the deamidase molecules from the three strains were the same.

**Electrophoretic Mobility**

The partially purified enzymes were concentrated to the same final volumes (the preparation from pncH9 contained 1.5 mg per ml of protein), and run on a pH 8.9 glycine-polyacrylamide gel with pH 8.3 buffer (17). A 5% gel proved to be the most effective. With the purified extract from pncH9 a strong band was observed 5.5 cm down the gel when the bromophenol band had moved 8.6 cm. This band was very weak with the wild type and was much more plentiful than in the wild type, and was enriched along with the enzyme. While this protein is very possibly the enzyme, attempts to demonstrate activity in any part of the gel were negative.

**Cotransduction of pncH Loci with pncA Locus**

Phage grown on the various pncH mutants were used to transduce E. coli W3889-nam11, the pncA, nadB double mutant. Cotransductants that received both pncA + and pncH were determined as colonies that appeared in 2 weeks on plates containing 30 mm nicotinamide as the sole nitrogen source (Table IV). Approximately 40% as many of these cotransductants appeared as did transductants on minimal agar plates supplemented with 0.1 mm nicotinamide as a vitamin source. These latter (total) are of two types: 51% pncA + and 49% nadB +. Hence, the cotransductants could be at most 51% of the total. The observed 40% is an underestimate, since growth was very slow on the nicotinamide plates. We conclude that more than 80% of the pncA + transductants also received the pncH gene. Thus, the two genes lie close together, appearing near about 35 min on the Taylor map where pncA has been located (Dickinson and Sundaram (6)).

**Discussion**

These results demonstrate again that the ability of a bacterium to produce a constitutive enzyme can be enhanced by mutation, 50-fold in the present case. This enhancement provides a great advantage for both enzyme production and purification. Yet the specific activity in our best mutant, 140 units per mg, is 100-fold less than the specific activity of an induced aliphatic deamidase from *Pseudomonas aeruginosa* (reported by Clarke (18)); therefore, further improvement should be obtainable.

Purification has resulted in a 200-fold increase in specific activity of nicotinamide deamidase from the wild type and also from two different mutants. The final specific activity of a preparation made from mutant pncH9 is 10,000 times the original wild type activity. The specific activity of this preparation is 3 X 10⁶ moles per mg per min.

The enzyme made by mutants pncH2 and pncH9 appear to be identical in structure to the wild type enzyme. No differences have been seen in the Km values, heat inactivation curves, or the pH optima. The presence of a strong protein band in the polyacrylamide gel containing partially purified enzyme from mutant pncH9 and a much weaker band at the same location in the gel containing enzyme from the wild type suggests that the mutation increases production of an unchanged protein; also possibly that the enzyme from pncH9 is fairly pure. We conclude that the mutation probably changes the rate of enzyme production, rather than the catalytic activity of the individual enzyme molecules.

Joshi and Handler (19) have purified a nicotinamide deamidase from the yeast *Torula cremoris*. The molecular weight of this enzyme is approximately 10⁵, its V max is 2000 moles per mg.

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per min which is 0.1 of the value reported here, and its $K_m$ is $1.4 \times 10^{-3}$ M which is one-fifth the $K_m$ of the E. coli enzyme.

Su, Albizati, and Chaykin (15) have purified a nicotinamide deamidase from rabbit liver. Their enzyme has a specific activity of 75 nmoles per mg per min, a value far lower than either of the above, and a $K_m$ far higher ($4 \times 10^{-2}$ M).

Curiously, this enzyme hydrolyzes a number of esters, including p-nitrophenyl acetate, 1000 times faster and with a 1000-fold lower $K_m$ than it does nicotinamide. It might be an esterase with a low activity on nicotinamide.

The genetic mechanism that regulates hyperconstitutivity is still a matter of speculation. In the present experiments, the high frequency of cotransduction, measured indirectly, for these mutants suggest that the regulatory apparatus and structural gene lie within a small fraction (less than 2%) of the E. coli genome. This proximity still allows several possible mechanisms, as discussed by Miller (20) and by Fraenkel and Banerjee (4). The first of these is a mutation in the promoter region adjacent to the structural gene. Such an "up" promoter might have an increased affinity for RNA polymerase and thereby increase the frequency of transcription of the corresponding structural gene. Examples of this mechanism might include 10- and 50-fold increased production of lac repressor production by E. coli mutants (20), the up to 100-fold hyperproduction of dihydrofolate reductase molecules by mutants of D. pneumoniae, with simultaneous changes in structure of the enzyme (2), and the 6-fold increase of glucose 6-phosphate dehydrogenase in E. coli (4).

An alternative of this mechanism is the conventional induction-repression model with the added requirement that the repressor protein has little or no affinity for an effector, as with arginine biosynthetic enzymes of E. coli B (21). Or the effector molecule might not be available within the cell, perhaps owing to a permeability barrier, as seems to be the case with histidase and urocanase of Salmonella typhimurium (22). Alternatively, the effector could always be present at a high concentration as proposed for constitutive production of a citrate transport system by Bacillus subtilis (23). Mutations might then alter the quantity or quality of the repressor, or the operator gene, or the effector concentration. Distinguishing between these various models seems difficult at present.

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