THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 246, No. 22, Issue of November 25, pp. 6792-6796, 1971
Printed in U.S.A.

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

(Received for publication, June 28, 1971)

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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.

* This research was supported by Public Health Service Research Grant CA-11595 from the National Cancer Institute.

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 *nad*B6 isolated by Dr. C. Yanofsky is a nicotinic acid auxotroph (5). A double mutant isolated by Dr. T. K. Sundaram (9) is *E. coli* W3889-*nam*11, a nicotinic acid auxotroph (*nad*B30), 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 KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of $(NH_4)_2SO_4$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, 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*-^{14}C]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 [^{14}C]nicotinamide to nicotinic acid. The 0.5 ml of standard reaction mixture contained 0.1 ml of 0.5 M potassium phosphate buffer, pH 7.0; 0.3 ml of 1.4 mM nicotinamide containing 2 μCi per ml of ^{14}C and 0.1 ml of enzyme preparation at a concentration chosen to yield less than 60% conversion. The ATP and $MgSO_4$ previously added (5) were found to be

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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 × 20 cm) (type K301R). The chromatograms were developed using n-butyl alcohol-10% ammonium hydroxide (19:1), in which nicotinamide has an R_F of 0.65 and nicotinic acid an R_F 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 mM 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 *pnc*H. Of six mutant cultures selected, one, *pnc*H6, was again mutagenized and selected in order to get another set of mutants.

Transduction—*E. coli* W3889-*nam*11, the *pnc*A, *nad*B double mutant, was transduced with P1 phage grown on the various *pnc*H mutants according to the procedure of Yanofsky and Lennox (14). Transductants were plated on three different media. If the phage introduces either *pnc*A or *nad*B a transductant can grow on minimal agar with 0.1 mM nicotinamide as a vitamin source. One must subtract the *nad*B transductants, which grow on minimal agar, from the total in order to determine the *pnc*A transductants. Transductants that received both the *pnc*A and *pnc*H genes (cotransductants) were scored after 1 to 2 weeks on minimal plates that contained 30 mM nicotinamide as the sole nitrogen source.

TABLE I

Nicotinamide deamidase activities of E. coli 2000α grown in different media

Concentration of addition to M63	Enzyme activity
μM	*nmoles/mg/dry cells/min*
None	2.9
Nicotinamide	
0.08	3.0
0.82	3.9
8.2	2.9
Nicotinic acid	
2.4	3.2
8.2	3.0
82.0	3.5
NAD	
0.2	2.8
2.0	2.9
20	3.0
NADP	
0.2	3.3
2.0	3.1
20	3.0
Nutrient broth	
—	3.4

RESULTS

Constitutive Production of Nicotinamide Deamidase

As Imsande 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 nad*B6 (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 *pnc*H 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 *pnc*H6 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 *pnc*H9 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 *pnc*H9 is given in Table III. A single colony of *E. coli pnc*H9 inoculated into 4 liters of nutrient broth was grown to the late log phase. The

TABLE II

Specific activities of crude extracts prepared from E. coli 2000α and pncH mutants

Strain	Relative activity
2000α	1.0
*pnc*H1	22
*pnc*H2	5
*pnc*H3	1
*pnc*H4	3
*pnc*H5	17
*pnc*H6	16
*pnc*H7	3
*pnc*H8	4
*pnc*H9	51
*pnc*H10	3
*pnc*H11	32
*pnc*H12	9
*pnc*H13	8

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TABLE III

Purification of nicotinamide deamidase from E. coli pncH9

	Total protein	Total activity	Recovery	Specific activity
	mg	*units*	%	*units/mg*
Sonicate	120	16,700	100	140
Protamine sulfate	110	22,100	132	200
$(NH_4)_2SO_4$	30	17,800	106	590
Acetone	2.0	9,000	50	4,500
Sephadex	0.14	3,800	23	27,200

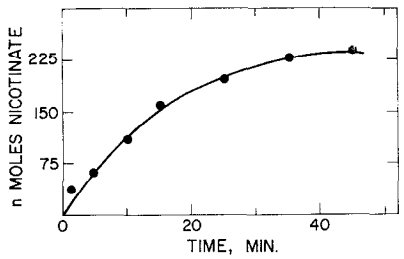

FIG. 1. Activity of purified enzyme from *pnc*H9 *versus* time of incubation. Assays were carried out under standard conditions using 0.4 μg of protein per assay.

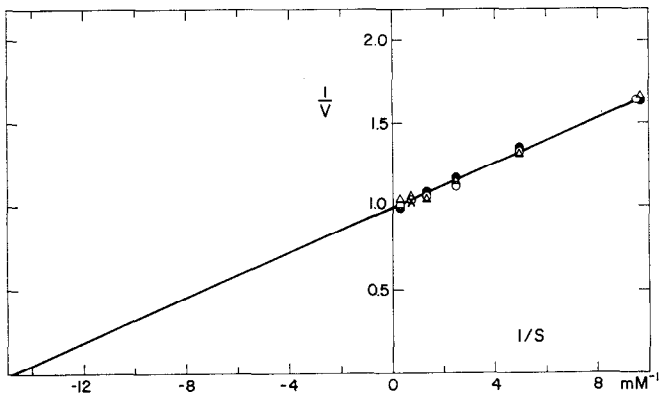

FIG. 2. Activity of purified enzymes *versus* substrate concentration. Assays were carried out under standard conditions except that the substrate concentrations were varied. The quantities of enzyme per assay were 0.6 μg for *pnc*H9 (●), 6.0 μg for *pnc*H2 (△), 22 μg for wild type (○). V_{max} values are normalized for better comparison; they are 2.9×10^4, 2.3×10^3, and 560 units per mg, respectively.

cells were harvested by centrifugation. The pellets were washed with chilled 0.5% 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° unless otherwise noted.

1. Sonic Oscillation—The frozen cells were resuspended in chilled 0.05 M potassium phosphate buffer, pH 7.0. Portions, 10-ml, were sonicated for 2 min in 30-s periods using a Branson sonifier at setting 7.0. The supernatants were collected after centrifugation at 13,000 × *g* for 15 min.

2. Protamine Sulfate Fractionation (15)—The pH of the extract was lowered to 6.0 by dropwise addition with stirring of chilled 1 N acetic acid. Then 0.88 ml per g of wet cells, of 1% protamine sulfate were added dropwise with continuous stirring during a 5-min interval. After centrifugation at 13,000 × *g* 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° was added dropwise with stirring to give 35% 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° were added dropwise, stirred for 15 min, and centrifuged at 22,000 × *g* for 30 min at −20°. 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° 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 × 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° in phosphate buffer at pH 7.0. It had a half-life of about 2 hours at 37°, 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° 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 *pnc*H9 and *pnc*H2 are compared. The K_m values were virtually identical at about 7×10^{-5} M, although the specific activities (V_{max}) varied over a 50-fold range.

The pH optimum was approximately 7.2, as measured in 0.1 M phosphate buffer at 0.2 pH unit intervals. The activity was nearly constant between pH 6 and 8. Similar results were obtained with the three partially purified preparations.

Molecular Weight Estimation

The elution volume on Sephadex G-100 of *pnc*H9 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

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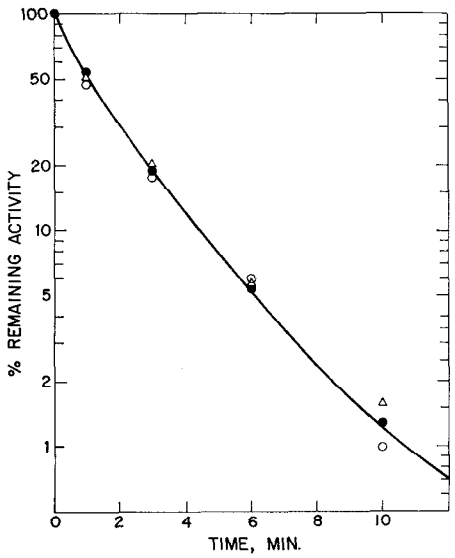

FIG. 3. Heat inactivation curves for purified enzymes. Two milliliters of each purified enzyme preparation were placed in a water bath at 55°, then samples of 0.2 ml were removed at the indicated times and placed on ice. The samples were then assayed in the usual manner; *pnc*H9 (●); *pnc*H2 (△); wild type (○).

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 *pnc*H9 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 *pnc*H9 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 preparation. Both preparations also had a similar, fainter band at 2.5 cm, and others that were much fainter. Thus, a very distinct difference was seen between the mutant and wild type purified extracts. The mutant contained a protein that 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 *pnc*H mutants were used to transduce *E. coli* W3889-*nam*11, the *pnc*A, *nad*B double mutant. Cotransductants that received both *pnc*A$^+$ and *pnc*H 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% *pna*A$^+$ and 49% *nad*B$^+$. 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 *pnc*A$^+$ transductants also received the *pnc*H gene. Thus, the two genes lie close together, appearing near about 33 min on the Taylor map where *pnc*A has been located (Dickinson and Sundaram (6)).[1]

TABLE IV

Transduction of pnc genes

The recipient was *E. coli* W3889-*nam*11 in all cases. The total frequency of transduction was 1 to 3 × 10^{-6} per phage in various experiments. The percentage of cotransduction was calculated by dividing the percentage of double transductants by 0.51, the mean fraction of *pnc*A transductants.

Donor	Total colonies	Hyper-production (*pnc*A, *pnc*H)	Deamidase$^+$ (*pnc*A)	Cotrans-duction
		%	%	%
None	36	0, —	17	—
2000α	1741	0.1, —	54	0.2, —
*pnc*H1	4147	43, 56	51	86, 114
*pnc*H2	3241	16, —	42	32, —
*pnc*H3	741	37, —	49	75, —
*pnc*H4	2038	49, 49	47	99, 99
*pnc*H5	1933	29, 42	50	58, 85
*pnc*H6	4488	35, 50	51	71, 100
*pnc*H7	1334	34, —	49	69, —
*pnc*H8	741	23, —	44	47, —
*pnc*H9	915	48, —	52	98, —
*pnc*H10	1471	37, —	52	76, —

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 *pnc*H9 is 10,000 times the original wild type activity. The specific activity of this preparation is 3 × 10^4 nmoles per mg per min.

The enzyme made by mutants *pnc*H2 and *pnc*H9 appear to be identical in structure to the wild type enzyme. No differences have been seen in the K_m 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 *pnc*H9 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 *pnc*H9 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 cremonis*. The molecular weight of this enzyme is approximately 10^4, its V_{max} is 2000 nmoles per mg

[1] G. J. Tritz and R. K. Gholson, personal communication.

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per min which is 0.1 of the value reported here, and its K_m is 1.4×10^{-5} 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 (4×10^{-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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J. Biol. Chem. 1971, 246:6792-6796.

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