Modification of Aspartate Before Its Condensation with Dihydroxyacetone Phosphate During Quinolinic Acid Formation in *Escherichia coli*

BRET M. STEINER,† JOHN T. HEARD, JR., AND GERALD J. TRITZ*

Department of Microbiology and Immunology, Kirksville College of Osteopathic Medicine, Kirksville, Missouri

A crude enzyme preparation from a nadA mutant of *Escherichia coli* was used to catalyze the conversion of [14C]aspartic acid into a precursor of quinolinic acid, a key intermediate in the biosynthesis of nicotinamide adenine dinucleotide.

The biosynthetic pathway leading to the formation of nicotinamide adenine dinucleotide (NAD) in *Escherichia coli* remains undefined. A defect in either one of two genetic loci (nadA, nadB) prevents the formation of quinolinic acid (QA), a key intermediate in the biosynthesis of NAD, from the known substrates aspartic acid and dihydroxyacetone phosphate (6). The nadA-coded enzyme precedes the nadB-coded enzyme in the pathway (5). The presence of these two enzymes argues for at least one intermediate in the formation of QA. Energetic considerations of the reactions argue in favor of more than one intermediate. For QA to be synthesized, two covalent bonds must be formed between aspartic acid (or a metabolite of aspartic acid) and dihydroxyacetone phosphate for ring closure, carbonyl reduction must occur, and three double bonds must be introduced into the ring structure (2). Because of the number of reactions that must occur, it is feasible to assume that multiple enzymes are required and a multiplicity of intermediates is formed.

In this paper we report the synthesis of a QA precursor from aspartic acid by a cell-free extract of a nadA mutant of *E. coli*. The presence of this compound supports the hypothesis that there is more than a single enzymatic step in the synthesis of QA from aspartic acid and dihydroxyacetone phosphate. Furthermore, we present definitive evidence that aspartic acid is modified before it condenses with dihydroxyacetone phosphate.

All bacterial strains were derivatives of *E. coli* K-12. Strain UTH4556 is a nadA auxotroph which also requires proline and histidine for growth. Strain UTH7042 is a nadC auxotroph.

The minimal medium of Davis and Mingioli (4) to which thiamine (2 mg/liter) and nicotinic acid (1.6 mM) were added was the basic growth medium. The repression medium contained 5 mM nicotinic acid but otherwise was the same as the standard growth medium. For the cultivation of strain UTH4556, the medium was additionally supplemented with 100 mg of histidine and 20 mg of proline per liter. The cell lines were carried on the appropriate minimal medium which had been solidified with 1.5% agar.

In a typical experiment, inoculum from the agar was added to 300 ml of minimal medium contained in a 1-liter flask. The medium was then incubated at 37°C with vigorous aeration on a New Brunswick reciprocal shaker for 24 h. At this point the cells were harvested by centrifugation in an International B-20A refrigerated centrifuge at 4°C for 10 min at a force of 7,500 × g. The supernatant was discarded, and the resulting pellet was suspended in a volume of 0.9% NaCl equal to four times the weight of the cells. This suspension was recentrifuged as previously described. The saline supernatant was decanted, and the resulting pellet was immediately frozen at −68°C.

Frozen cells for in vitro experiments were partially thawed at ambient temperature and then suspended in a volume of 0.05 M phosphate buffer at pH 8.0 (7.63 g of K_2HPO_4 and 0.85 g of KH_2PO_4 per liter) equal to four times the weight of the cells. The cells were exposed to ultrasonic waves in an Artek sonic dismembrator model 150 at a setting of 80 W for five 4-min intervals. During this period, the cells were contained in a centrifuge tube which was in an ice bath. Between each period of sonication the sonic probe was cooled by immersion in ice water. Cellulose debris was removed by centrifuging at 7,500 × g for 10 min. The resulting cell lysate was used as a source of enzyme for the in vitro synthesis of QA and its precursors.

QA and its precursors were synthesized from radiolabeled and unlabeled fructose-1,6-diphosphate (as a source of dihydroxyacetone phosphate) or radiolabeled and unlabeled aspartic
acid, or both, by the method previously described (2). The only modification was an increase (to 10 μCi) in the amount of labeled substrate.

[U-14C]aspartic acid and [U-14C]fructose-1,6-diphosphate were obtained from the Amersham Corp. Each of these products contained impurities equal to less than 2% of the total disintegrations in the sample as determined through separation by ion-exchange chromatography (described above) and quantitation in a liquid scintillation counter. Flavin adenine dinucleotide was acquired from the Eastman Corp. 1,4-Bis[2-(5-phenyloxazolyl)]benzene and 2,5-diphenyloxazole were purchased from the Fisher Scientific Co. Other chemicals were of the highest quality available and were acquired from Sigma, Aldrich, and Baker chemical companies.

A cell-free extract of a nadA mutant was used to catalyze the conversion of aspartic acid into presumed precursors of QA. These metabolites were then subjected to separation by ion-exchange chromatography. The resulting profile of radioactivity is illustrated in Fig. 1a. Peak F

![Diagram](http://jb.asm.org/)

**Fig. 1.** Elution profile of radiolabeled compounds synthesized by a nadA mutant of E. coli. Before chromatography the sample was prepared by precipitating the protein from the in vitro reaction mixture with 0.3 ml of 1.5 N perchloric acid. The mixture was then centrifuged for 10 min at 7,500 × g, and the precipitate was discarded. The perchloric acid was in turn precipitated by the addition of 0.2 ml of 2.5 N KOH. The mixture was again centrifuged for 10 min at 7,500 × g, and the precipitate was discarded. The pH of the supernatant was adjusted to neutrality with 4 N acetic acid. A 1-ml portion of this preparation was then subjected to ion-exchange chromatography. The ion exchange resin was Dowex-1-chloride X-8. The resin was prepared for chromatography by the method of Cooper (3) with a slight modification; the resin was washed with 0.1 M ammonium acetate starting buffer until pH stability, instead of water as in the method of Cooper. The prepared resin was packed in a 1-cm-diameter column to a height of 20 cm. The sample was layered on the column, and the column was washed with 30 ml of 0.1 M ammonium acetate. An ammonium acetate gradient was then applied (0.1 to 1.5 M) by using 18 ml of each ammonium acetate concentration. After the gradient, the column was successively washed with 30 ml of 2 N acetic acid and 40 ml of 2 N HCl to remove any tightly bound materials that would not elute with the gradient. Samples of 3 ml were collected of all of the column washings. These samples were then analyzed for radioactivity by liquid scintillation spectrophotometry. A fraction of 100 μl was taken from each tube and placed in a scintillation vial containing 7.5 ml of scintillation fluid. The scintillation fluid contained (per liter): 600 ml of toluene, 400 ml of 95% ethanol, 4 g of 2,5-diphenyloxazole, and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene. The radioactivity in each sample was quantitated in a Beckman LS-100 liquid scintillation counter.
represents aspartic acid, and this peak of activity almost completely disappears as the enzyme reaction proceeds. Concomitant with the disappearance of peak F is the appearance of peak G. Peaks A and D represent impurities in the commercial \([^{14}C]\text{aspartate}\) as well as, perhaps, extraneous products of aspartic acid metabolism; each of these peaks represents multiple compounds. Evidence that peak G contained a compound related to the biosynthesis of NAD was obtained by showing that an enzyme necessary for the biosynthesis of peak G material was repressed by 5 mM nicotinic acid (Fig. 1b). Under repressed conditions only peak G disappears from the profile of radioactive materials.

Early intermediates in the QA biosynthetic pathway might be expected to incorporate label from a single source, either aspartic acid or fructose-1,6-diphosphate, whereas more immediate precursors of QA would be expected to incorporate label from both sources. Since the available evidence indicates that the nadA gene codes for an enzyme in the early part of the pathway, it was presumed that the nadA mutant was blocked at an initial step in the synthesis of QA. If this were so, then the material in peak G would not necessarily be expected to incorporate label from fructose-1,6-diphosphate (as a source of dihydroxyacetone phosphate). Figure 1c and d indicate that the nadA mutant does not incorporate label from fructose-1,6-diphosphate into peak G. To rule out the trivial explanation that the nadA mutant lacked the ability to convert fructose-1,6-diphosphate into dihydroxyacetone phosphate, it was demonstrated that the crude enzyme preparation from the nadA mutant and commercially acquired aldolase exhibited identical enzymatic activity toward fructose-1,6-diphosphate, i.e., the enzymatic products were the same.

The definitive proof that peak G contains a precursor of QA is the evidence that the material can be converted to QA. Peak G was subjected to fractionation by several chromatographic procedures, and in all cases it migrated as a single peak. The following chromatographic systems were employed: (i) low-pressure liquid chromatography on a Dowex 1 \(\times\) 8-400 acetic anion-exchange column with elution by 0.2 N sodium citrate; (ii) low-pressure liquid chromatography on a Dowex 50 \(\times\) 8-100 hydrogen cation-exchange column with elution by a gradient of HCl from 0 to 2 N; (iii) high-pressure liquid chromatography with a Waters model ALC/GPC chromatography, a \(\mu\)-Bondapak C\(_{18}\) reverse-phase column, and a solvent of 0.005 M tetrabutyl ammonium phosphate (pH 7.5). Therefore, peak G represented a relatively pure substance. This material was used as the substrate for the QA-biosynthetic enzymes in a nadC mutant. The nadC genetic block occurs after the synthesis of QA and prevents the conversion of QA into nicotinic acid mononucleotide; QA accumulates in these mutants. In this system, QA was synthesized from the peak G material (Table 1). Furthermore, this conversion occurred at a more rapid rate than the synthesis of QA from aspartate.

The metabolite which we have isolated appears to be an early intermediate in the biosynthesis of QA. This finding supports the hypothesis of Wicks et al. (7) that aspartic acid is modified before it condenses with dihydroxyacetone phosphate; however, our experiments indicate that the aspartate metabolite is more stable than Wicks et al. postulate. The presence of this compound argues for the existence of more than one intermediate and more than two enzymes in QA biosynthesis. Presently we picture the pathway as the following series of reactions:

\[
\text{Aspartic acid} \rightarrow X \text{ (represented by peak G)} \rightarrow Y \\
Y + \text{dihydroxyacetone phosphate} \xrightarrow{\text{nadA-coded enzyme}} Z \\
Z \xrightarrow{\text{nadB-coded enzyme}} \text{quinolinic acid}
\]

The above scheme supposes the existence of a compound, Z, which incorporates label from both aspartic acid and dihydroxyacetone phosphate. Actually, there may be more than one compound which incorporates label from both sources. We have previously reported evidence of the existence of such condensation products as well as evidence to support the existence of two modified aspartate compounds, X and Y (2).

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<th>Table 1. Efficiency of QA production*</th>
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<td>Substrate</td>
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*Quinolinic acid (QA) production was measured over a 1-h period. Further incubation for up to 5 h did not significantly change the results. QA was assayed by the method of Chandler and Gholson (1). cpm, Counts per minute.
LITERATURE CITED


