Vol. 84, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 30, 1978 Pages 928-935

SYNTHESIS OF QUINOLINATE FROM D-ASPARTATE IN THE MAMMALIAN LIVER-ESCHERICHIA COLI QUINOLINATE SYNTHETASE SYSTEM*+

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Received September 11,1978

Summary: Two proteins (A and B) from Escherichia coli are required for the synthesis of the NAD precursor quinolinate from aspartate and dihydroxyacetone phosphate. Mammalian liver contains a FAD linked protein which replaces E. coli B protein for quinolinate synthesis. D-aspartic acid but not L-aspartic acid is a substrate for quinolinic acid synthesis in a system composed of the B protein replacing activity of mammalian liver and E. coli A protein. In contrast the E. coli B protein-E. coli A protein quinolinate synthetase system requires L-aspartic acid as substrate. The previous report that L-aspartate was a substrate in the liver-E. coli system was due to contamination of commercially available [^{14}C]L-aspartate with [^{14}C]D-aspartate. These and other observations suggest that liver B protein is D-aspartate oxidase and E. coli B protein is L-aspartate oxidase.

Escherichia coli contains two proteins (designated A and B) which together convert L-aspartate and DHAP† into the NAD precursor QA in an FAD requiring reaction (1)(2). We have recently reported that mammalian liver contains a protein which fully replaces the B protein activity of this E. coli QA synthetase system (3). This finding of B protein activity in mammalian liver was puzzling since, although QA is well established as a precursor of NAD in this tissue (4), mammals do not form QA from aspartate but from tryptophan (5). Moreover we were able to detect only traces of activity of the A protein component of QA synthetase in liver (3).

Originally we reported (3) that L-aspartate is a substrate for QA synthesis in the hybrid liver-E. coli QA synthetase system. However, further investigation of this hybrid system has established that D-aspartate, but not L-asparatate, is incorporated into QA when liver is the source of B protein

*Journal article #3507 of the O.S.U. Agricultural Experiment Station.

+This work was supported in part by grant # PCM 77-18814 from the National Science Foundation.

†Abbreviations used QA-quinolinic acid, DHAP dihydroxyacetone phosphate, GOT-glutamic oxaloacetic transaminase.

0006-291X/78/0844-0928$01.00/0

activity. The apparent incorporation of L-aspartate (3) was due to contamination of commercially available preparations of "[U-^{14}C]L-aspartate" with varying amounts of [^{14}C]D-aspartate. A D-aspartate oxidase has been previously described in mammalian kidney (6). The finding that the B protein replacing activity of mammalian liver is specific for D-aspartate, together with the observation that both the liver and E. coli B proteins produce an unstable compound from aspartate which can be converted to QA by E. coli A protein in the presence of DHAP (7), suggest that both the liver and bacterial B proteins have aspartate oxidase activity and cast new light on the mechanism QA of synthesis in E. coli.

Experimental Methods:

Materials. Reagents were purchased from the following sources: "[U-^{14}C]L-aspartic acid" (New England Nuclear and ICN Inc.); [4-^{14}C]DL-aspartic acid, [U-^{14}C]D-fructose 1,6-diphosphate (New England Nuclear); N,N-bis-2-hydroxymethyl-glycine (Bicine), quinolinic acid, dihydroxyacetone phosphate (dimethyl ketal), FAD, D-fructose 1,6-diphosphate, L- and D-aspartic acid, catalase (720 units/mg), glutamic oxaloacetic transaminase (100 units/mg), oxaloacetic acid (Sigma); 2,4-dinitrophenylhydrazine (Kodak); potassium sodium tartaric acid (J. T. Baker); sodium pyrophosphate (Fischer); Protein assay kit, Dowex resisn (Bio-Rad); DEAE-cellulose (Whatman); and Sephadex G-75 (Pharmacia). The dihydroxyacetone phosphate dimethyl ketal was hydrolyzed immediately before use according to the manufacturer's directions. [4-^{14}C]DL-aspartic acid was purified by Dowex 1x8 (acetate form, 100-200 mesh) column chromatography. The aspartic acid fraction was eluted with 0.5N acetic acid and then was taken to dryness on a rotary evaporator. The residue was carefully dissolved in distilled water and adjusted to pH 6 with KOH.

Purification of D-aspartic acid oxidase. The D-aspartic acid oxidase used in this experiment was partially purified from beef kidney by the method of Rinaldi (6). The steps were a) Heating at 60°C at pH 5 for 15 min in the presence of 10 mM sodium potassium tartaric acid, b) 30-40% ammonium sulfate fractionation, c) Ca-phosphate gel treatment and d) Sephadex G-75 column chromatography. The final enrichment was about 250 fold over the crude extract. The activity of D-aspartic acid oxidase was measured by a modification of the method of Davies and Johnston (8). The assay was performed open to the air at 37°C for 30 min in a shaking water bath. The reaction mixture consisted of 76 mM sodium pyrophosphate buffer, OH 8.3, 10 µM FAD, 80 µg of catalase, and 20 mM D-aspartic acid, and up to 0.3 ml of enzyme in a final volume of 1.0 ml. The enzyme was preincubated with FAD for 15 min before initiation of the reaction by the addition of substrate. Reaction was stopped with 0.2 ml of 50% trichloroacetic acid and centrifuged. Product oxaloacetic acid was determined on a 1.0 ml portion of the supernatant by addition of 0.1 ml of 0.2% 2,4-dinitrophenylhydrazine in 2N HCl, incubation at 37°C for 30 min, and then reading the absorption at 415 nm (9) in a spectrophotometer 10 min after the addition of 4.0 ml of 1.25N NaOH. A substrate blank was run as control.

Preparation of [4-^{14}C]L- and D-aspartic acids. These isomeric ^{14}C-aspartic acids were prepared from [4-^{14}C]DL-aspartate by destruction of the D-isomer with D-aspartic acid oxidase and the L-isomer with GOT. Twenty μCi of [4-^{14}C]DL-aspartic acid (spec. act. 23.57 Ci/mol) was incubated at 37°C for 3 hours with 0.39 mg of D-aspartic acid oxidase, partially purified from beef kidney by the method of Rinaldi (6), 0.1 nmole of FAD, and 500 nmoles of bicine buffer (pH 8.0) in a total volume of 4.0 ml.

The reaction was stopped by adding 0.5 ml of 15% $HClO_4$. The precipitated protein was compacted by centrifugation and the supernatant solution was neutralized with KOH. After centrifugation, the supernatant solution was applied to a Dowex 1 x 8 (acetate form, 100-200 mesh) column (0.8 x 20 cm) and was eluted with 0.5 M acetic acid. The aspartic acid fraction was taken to dryness on a rotary evaporator. The residue was carefully dissolved in 2 ml of distilled water and adjusted to pH 6 with KOH. The recovery of radioactivity was 43%. ^{14}C-aspartic acid obtained using the method as described above was not destroyed by D-aspartic acid oxidase, but was completely destroyed by GOT. These findings indicate that the ^{14}C-aspartic acid obtained was the L-form. The GOT treatment was performed at 37°C for 6 hours with the reaction mixture containing 20 μCi of [4-^{14}C]DL-aspartic acid, 50 units of GOT, 5 μmoles of α-ketoglutaric acid, and 500 μmoles of bicine buffer (pH 8.0) in a total volume of 4.0 ml. The radioactive aspartic acid was isolated using the method described above. The recovery was 44%. The ^{14}C-aspartic acid obtained was destroyed by D-aspartic acid oxidase but not by GOT.

Growth of bacteria. The nad A mutant (PA-2-18) and nad B mutant (SB-16) of E. coli K-12 used, and their growth conditions, were previously described (1).

Preparation of A and B. proteins. The purification procedures for the A protein were similar to those previously described (1). The E. coli B protein used in these experiments was purified from the nad A mutant (PA-2-18) by 2% protamine sulfate precipitation and 0-50% ammonium sulfate fractionation. Calf liver was homogenized for 5 min at 4°C in a Waring blender with 3 volumes of 0.05 M KPO_4 (pH 8.0). The homogenate was centrifuged for 30 min at 30,000 x g. The 35-55% ammonium sulfate fraction of the supernatant solution was used as calf liver B protein for these experiments.

Assay method for quinolinic acid synthetase. The standard reaction mixture contained 0.25 μmole of [4-^{14}C]D- or L-aspartic acid (spec. act. 2 Ci/mole), 0.25 μmole DHAP, 0.01 μmole FAD, 50 μmoles of bicine buffer (pH 8.0) and protein fractions in a total volume of 0.5 ml. The reaction mixture was incubated at 25°C for 10 min and was stopped by the addition of 0.5 ml of 15% $HClO_4$. Then 0.5 ml of 2.0 mM quinolinic acid was added. The denatured protein was compacted by centrifugation and the supernatant solution neutralized with KOH. [^{14}C]QA was isolated by the two column ion exchange chromatography system previously described (10). After measuring the absorbance at 268 nm, the radioactivity was determined by counting with a Beckman scintillation spectrometer, using Packard Instagel, and the yield of quinolinic acid calculated. Protein concentration was determined by the Bio-Rad Protein Assay (11).

Results and Discussion: The first indication that L-aspartate might not be the true substrate for QA synthesis was provided by studies of the time course of the conversion of aspartate to an unidentified radioactive compound by partially purified liver B protein. This compound is formed instead of QA when the A protein is omitted from the reaction mixture. It is easily

separated from both QA and aspartate in our column QA assay system (10) and is probably a degradation product of an unstable intermediate in QA synthesis. Initial rates of synthesis of this compound were rapid but dropped to zero after 5% of the starting "[U-^{14}C]L-aspartate" was converted. Addition of fresh enzyme did not lead to further synthesis but addition of more "[U-^{14}C]L-aspartate" led to another round of synthesis again amounting to about 5% of the ^{14}C-aspartate added (data not shown). These observations led us to suspect that a radioactive contaminant in the [U-^{14}C]L-aspartate rather than L-aspartate itself was the true substrate for liver B protein. Since the ^{14}C-aspartate had been purified on a Dowex-1 acetate column shortly before use, D-aspartate seemed to be the most likely contaminant. We therefore undertook the following series of experiments in order to establish whether D-aspartate is the precursor of QA in the hybrid liver-*E. coli* QA synthetase system.

In the first experiment, the results of which are shown in Table I, the substrate specificities of D- and L-aspartic acids for the synthesis of QA in the *E. coli* QA synthetase systems were studied. In this experiment, QA synthesis was measured by the incorporation of [U-^{14}C]DHAP (formed *in situ* by the action of aldolase on [U-^{14}C]fructose 1,6-bisphosphate) into ^{14}C-QA. We have previously established that DHAP is the precursor for carbons 4, 5 and 6 of QA in the QA in the *E. coli* system (12).

As shown by the data presented in Table I, the *E. coli* B protein-*E. coli* A protein QA synthetase system incorporated [U-^{14}C]DHAP into ^{14}C-QA when L-aspartate was provided as the other substrate but not when D-aspartate was added. On the other hand when the liver B protein-*E. coli* A protein QA synthetase system was used [U-^{14}C]DHAP was incorporated into ^{14}C-QA only when the D isomer of aspartate was present in the reaction mixture.

Further evidence that the QA synthetase system containing liver B protein is specific for the D isomer of aspartic acid is provided by the results shown in Table 2. Commercially available [4-^{14}C]DL-aspartic acid was

Table I

Effect of Aspartate Isomer on Incorporation of [U-^{14}C]D-Fructose 1,6-bisphosphate into QA in the *E. coli* and Liver-*E. coli* QA Synthetase Systems

Enzyme System	L-aspartic acid	D-aspartic acid
	QA nmoles/10 min	
E. coli B + *E. coli* A	3.60	<.01
Calf Liver B + *E. coli* A	<.01	1.30

The reaction mixtures contained 0.2 μmole of [U-^{14}C]D-fructose 1,6-bisphosphate (spec. act. 5 Ci/mole), 1 unit of aldolase, 0.25 μmole of L- or D-aspartic acid, 0.01 μmole FAD, 50 μmoles of bicine buffer (pH 8.0), and protein fractions in a total volume of 0.5 ml. The assays contained 0.94 mg of *E. coli* A protein Sephadex fraction, and 0.14 mg of *E. coli* B protein ammonium sulfate fraction or 0.53 mg of calf liver B protein ammonium sulfate fraction. Assays were carried out in duplicate using the standard method described in Methods.

Table II

Incorporation of [4-^{14}C]D- and L-Aspartic Acids into QA in the *E. coli* and Liver-*E. coli* QA Synthetase Systems

Enzyme System	*E. coli* B-*E. coli* A	Calf liver B-*E. coli* A
	Incorporation into QA (dpm/reaction mixture)	
[4-^{14}C]DL-aspartic acid	85,350	52,170
[4-^{14}C]L-aspartic acid	80,340	100
[4-^{14}C]D-aspartic acid	1,020	50,190

The reaction mixtures for the assay of QA biosynthesis contained either 10^6 dpm of [4-^{14}C]DL-aspartic acid or 5 x 10^5 dpm of [4-^{14}C]D-aspartic acid or [4-^{14}C]L-aspartic acid, 0.25 nmole of DHAP, 0.01 nmole of FAD, 50 nmoles of bicine buffer (pH 8.0), and the proteins indicated in a total volume of 0.5 ml. The assays contained 0.94 mg of *E. coli* A protein Sephadex fraction and 0.14 mg of *E. coli* B protein or 1.06 mg of calf liver B protein ammonium sulfate fraction. The assays incubated at 25°C for 10 min. Values are the average of duplicate assays.

resolved into [4-^{14}C]L-aspartic acid by destruction of the D isomer with beef kidney D-aspartic acid oxidase and into [4-^{14}C]D-aspartic by destruction of the L-isomer with GOT. Following these enzymatic treatments the resultant

D and L aspartic acids were isolated by Dowex-1 acetate column chromatography. When the [4-^{14}C]DL-aspartate was the radioactive substrate, ^{14}C-QA was formed by both the E. coli and the liver-E. coli QA synthetase systems (Table 2). However, when [4-^{14}C]L-aspartate was the radioactive substrate only the pure E. coli system formed ^{14}C-QA and when [4-^{14}C]D-aspartate was the radioactive substrate ^{14}C-QA was formed in appreciable amounts only by the hybrid liver B protein-E. coli A protein QA synthetase system. Similar results were obtained when the "[U-^{14}C]L-aspartate" preparations were resolved with isomer specific enzymes. D-aspartic acid oxidase treated "[U-^{14}C]L-aspartate" was incorporated into ^{14}C-QA only in the pure E. coli system. Untreated "[U-^{14}C]L-aspartate" was incorporated into ^{14}C-QA in both systems (data not shown). Recovery data indicated that the NEN product contained approximately 5-7% D-isomer and that the ICN [U-^{14}C]L-aspartate was about 50% D-isomer.

Further evidence for the specificity for the D and L isomers of aspartate of these two QA synthesis systems is provided by the dilution of incorporation of [4-^{14}C]DL-aspartate into ^{14}C-QA upon addition of ^{12}C-D and L aspartate. As shown in Figure 1A, addition of unlabeled L-aspartate to the E. coli QA synthetase system results in a large decrease in ^{14}C incorporation into QA from [4-^{14}C]DL-aspartate while unlabeled D-aspartate has very little effect on this incorporation. In contrast, as shown in Figure 1B, unlabeled L-aspartate in the liver-E. coli system has very little effect on incorporation of [4-^{14}C]DL-aspartate into ^{14}C-QA while unlabeled D-aspartate drasticly decreases ^{14}C-QA formation.

The optical specificity demonstrated above, together with the previous observation of D-aspartase oxidase activity in mammalian tissues and our recent observations that the B proteins from E. coli and liver form an unstable, dialyzable intermediate from "[U-^{14}C]L-aspartate" which is converted to ^{14}C-QA by the A protein in the presence of DHAP (7) lead to the following hypothetical formulation of QA synthesis in E. coli and the liver-

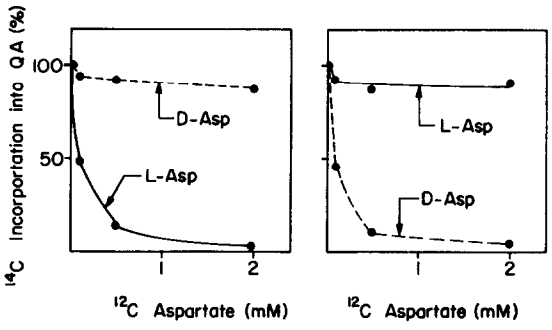
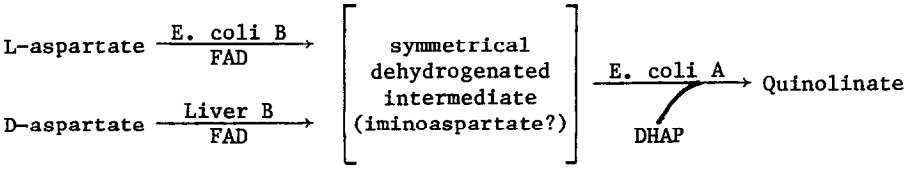


Fig. 1: Effect of addition of [^{12}C]L- and D-aspartate on the incorporation of [^{14}C]DL-aspartic acid into QA in the E. coli and liver-E. coli QA synthetase systems.

The reaction mixture contained 1.0 µCi of [4-^{14}C]DL-aspartic acid (spec. act. 23.57 Ci/mol), 0.25 nmoles of DHAP, 0.01 nmole of FAD, 50 nmoles of bicine buffer (pH 8.0), L- or D- aspartic acid in the range 0-2 mM, and protein fractions in a total volume of 0.5 ml. Assays contained 0.94 mg of E. coli A protein Sephadex fractions and 0.14 mg of E. coli B protein or 0.53 mg of calf liver B protein ammonium sulfate fraction. The assays were incubated at 25°C for 10 min. With no addition of ^{12}C aspartic acids the incorporation into ^{14}C-QA of [4-^{14}C]DL-aspartic acid was 150,630 dpm in the E. coli system and 63,080 dpm in the liver-E. coli system.

E. coli QA synthetase system:



The partially purified B protein fraction from liver exhibits D-aspartase oxidase activity as determined by both the 2,4-dinitrophenylhydrazine assay (8) and a coupled malic dehydrogenase assay. Work is now underway to unequivocally establish whether the same protein has D-aspartate oxidase activity and QA synthetase B protein activity.

The finding of varying amounts of [^{14}C]-D-aspartate in commercial preparations of "[U-^{14}C]L-aspartate" could be due to the preparation of this compound by acid hydrolysis of algal protein since it has been reported that heating L-aspartate to 170-180° in excess HCl results in recemization (13).

References:

1. Suzuki, N., Carlson, J., Griffith, G., and Gholson, R. K. (1973) Biochim. Biophys. Acta 304, 309-315.

2. Griffith, G. R., Chandler, J. L. R., and Gholson, R. K. (1975) Eur. J. Biochem. 34, 239-245.
3. Sakakibara, S., Wicks, F. D., and Gholson, R. K. (1977) Biochem. Biophys. Res. Commun. 76, 158-166.
4. Gholson, R. K., Ueda, I., Ogasawara, N., and Henderson, L. J. (1964) J. Biol. Chem. 239, 1208-1214.
5. Henderson, L. M., and Hirsch, H. M. (1949) J. Biol. Chem. 181, 667-675.
6. Rinaldi, A. (1971) Enzymol. 40, 314-321.
7. Wicks, F. D., Sakakibara, S., and Gholson, R. K. (1978) J. Bacteriol. (In Press).
8. Davis, L. P. and Johnston, G. A. R. (1975) J. Neurochem. 25, 299-304.
9. Katsuki, H., Yoshida, T., Tanegashima, G., and Tanaka, S. (1971) Analyt. Biochem. 43, 349-356.
10. Chandler, J. L. R. and Gholson, R. K. (1972) Anal. Biochem. 48, 529-535.
11. Bio-Rad Technical Bulletin 1051.
12. Wicks, F. D., Sakakibara, S., Gholson, R. K., and Scott, T. A. (1977) Biochim. Biophys. Acta, 500, 213-216.
13. Greenstein, J. P. and Winitz, M. (1961) Chemistry of the Amino Acids. vol. 3. John Wiley and Sons, Inc., New York. p. 1876.