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Replacement of the B Protein Requirement of the E. coli Quinolinate Synthetase System by Chemically-Generated Iminoaspartate[+x]

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Summary: Two proteins (A and B) from Escherichia coli are required for in vitro synthesis of the NAD^+ precursor, quinolinate, from L-aspartate and dihydroxyacetone phosphate. The requirement for B protein and L-aspartate in this system can be replaced by millimolar concentrations of oxaloacetate and ammonia if they are added together. This finding supports the concept that the B protein (L-aspartate oxidase) functions to form iminoaspartate which is condensed with dihydroxyacetone phosphate by the A protein to form quinolinate.

Quinolinic acid (QA) is a precursor of the pyridine nucleotides in a wide variety of organisms (1). In E. coli QA is synthesized from L-aspartate and dihydroxyacetone phosphate (DHAP) (2,3). Two enzymes, A and B, are required for this synthesis (3). The B protein catalyzes the FAD- and oxygen-dependent conversion of L-aspartate to an unstable intermediate which is condensed with DHAP by the A protein to form QA (4). We have recently shown that the B protein is L-aspartate oxidase and have postulated that the unstable product of this enzyme reaction is iminoaspartate (5,6). However, others have proposed that several enzymes, in addition to A and B, are required for QA synthesis in E. coli (7) and that A protein acts before B protein in this pathway.

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Herein we present evidence that a mixture of 5 mM oxaloacetate (OAA) and 5 mM $(NH_4)_2SO_4$ can completely replace both L-aspartate and B protein in the E. coli QA synthetase system. This further supports our concept of QA synthesis in E. coli as a two-step process in which the formation of iminoaspartate from L-aspartate by B protein is followed by the condensation of DHAP and iminoaspartate catalyzed by A protein.

Materials. Reagents were purchased from the following sources: "L-[U-^{14}C]aspartic acid" (ICN Inc.); D-[U-^{14}C]fructose-1,6-bisphosphate (FDP), (New England Nuclear); N,N-bis(2-hydroxyethyl)glycine (Bicine), quinolinic acid, dihydroxyacetone phosphate (dimethyl ketal), FAD, D-fructose-1,6-bisphosphate, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), L-aspartic acid, aldolase (rabbit muscle), catalase (720 units/mg), glutamic-oxaloacetic transaminase (100 units/mg), oxaloacetic acid (Sigma); citric acid monohydrate and Tris(hydroxymethyl)aminomethane (Fisher); protein assay kit, Dowex resins (Bio-Rad); and Sephadex G-75 (Pharmacia). The dihydroxyacetone phosphate dimethyl ketal was hydrolyzed immediately before use according to the manufacturer's directions.

Methods. Preparation of pure L-[U-^{14}C]aspartic acid, growth and harvesting of E. coli nadA and nadB mutants, purification of A and B proteins and assay for formation of [^{14}C]quinolinic acid were carried out by the methods previously described (6,8).

Results and Discussion: We have shown that iminoaspartate spontaneously decomposes to form OAA with a half-life, dependent on pH and temperature, of about 2½ min at pH 8.0 and 25°C (5,8). However, despite the large K_{eq} of this reaction, we thought it possible that sufficient iminoaspartate might be formed from high concentrations of OAA and NH_4^+ to allow the synthesis of [^{14}C]QA from [^{14}C]DHAP by the A protein of the E. coli QA synthetase system. As shown by the data presented in Table I that is indeed the case.

In the presence of A and B proteins, L-aspartate, and FAD, [^{14}C]DHAP (generated in situ from [^{14}C]FDP by aldolase) is incorporated into [^{14}C]QA (line 1). However, when L-aspartate and B protein are omitted no [^{14}C]QA is formed. Addition of 2.5 mM OAA or 2.5 mM $(NH_4)_2SO_4$ to A protein separately does not result in [^{14}C]QA formation, but when both OAA and NH_4^+ are present in the reaction mixture [^{14}C]DHAP is converted to [^{14}C]QA only slightly less efficiently than when B protein and L-aspartate are present. The addition of OAA and NH_4^+ to the reaction mixture containing B protein and L-aspartate

Table I

Replacement of B protein requirement for QA synthesis by an iminoaspartate-generating system

[^{14}C] Substrate	ENZ A µg	ENZ B mg	OTHER ADDITIONS	[^{14}C]QA formed (nmoles/5 min)
DHAP	.60	2.7	L-aspartate, FAD	1.95
DHAP	1.20	0	none	< .01
DHAP	1.20	0	$(NH_4)_2SO_4$	< .01
DHAP	1.20	0	OAA	< .01
DHAP	.60	0	$(NH_4)_2SO_4$, OAA	1.47
DHAP	1.20	0	$(NH_4)_2SO_4$, OAA	2.75
DHAP	.60	2.7	L-aspartate, FAD, NH_4^+, OAA	1.87
L-Aspartate	.60	2.7	DHAP, FAD	1.42
L-Aspartate	.60	2.7	DHAP, FAD, NH_4^+, OAA	0.45

The reaction mixtures all contained: E. coli A protein purified through the Sephadex G-75 step; Bicine (pH 8.0) 50 µmoles; FDP, 0.25 µmoles; aldolase, 3.5 units to generate DHAP; and, when indicated, E. coli B protein purified through the sodium citrate step; L-aspartate, 0.25 µmole; FAD, 0.01 µmole; OAA, 2.5 µmoles; $(NH_4)_2SO_4$, 2.5 µmoles in a total volume of 0.5 ml. When indicated L-[U-^{14}C]aspartate or D-[U-^{14}C]FDP were substituted for the unlabeled compounds. Incubation was carried out for 5 min at 25° and the reaction was stopped and [^{14}C]QA formation determined as described in Nasu, et al., (6).

does not inhibit the conversion of [^{14}C]DHAP to [^{14}C]QA. However, when L-[^{14}C]aspartate is the source of radioactivity being incorporated into QA, addition of OAA and NH_4^+ to the reaction mixture greatly reduces the incorporation of L-[^{14}C]aspartate into [^{14}C]QA, probably due to dilution of the pool of [^{14}C]iminoaspartate, formed from L-[^{14}C]aspartate by the B protein, with unlabeled iminoaspartate, formed from OAA and NH_4^+.

Experiments were then carried out to establish the optimum concentrations of OAA and NH_4^+ for QA synthesis. Figure 1A shows the effect on QA synthesis of varying OAA concentration from 0.5 to 10 mM in the presence of 2.5 mM $(NH_4)_2SO_4$. Figure 1B shows the effect of varying NH_4^+ concentration

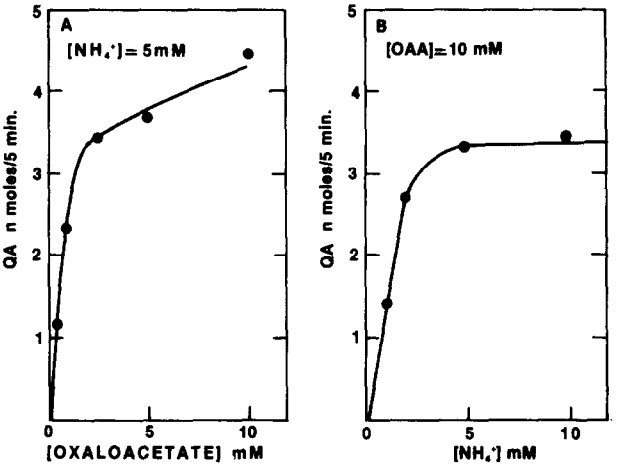


Fig. 1: Formation of [4-^{14}C]quinolinate from [^{14}C]DHAP as a function of oxaloacetate and ammonium sulfate concentrations.

The reactions were carried out as described in Table I with the exception that a higher specific activity A protein preparation was used. All reaction mixtures contained 0.5 mM D-[U-^{14}C]fructose-1,6-bisphosphate and 3.5 units of aldolase to generate [U-^{14}C]DHAP and the reaction mixtures contained the concentrations of oxaloacetate and ammonium sulfate indicated.

from 1.0 to 10.0 mM in the presence of 10 mM OAA. On the basis of these results most subsequent experiments were carried out with 10 mM OAA and 5 or 10 mM NH_4^+.

Although our two-column assay procedure is highly specific for the isolation of QA (9), replacement of the B protein and L-aspartate requirements of our QA synthetase system by OAA and NH_4^+ represented a substantial change from standard conditions. This made it necessary to verify that the radioactive product formed from [^{14}C]DHAP in our new system was actually [^{14}C]QA. We therefore carried out a four-fold sized reaction containing 10 mM OAA and NH_4^+, [^{14}C]DHAP (generated *in situ* from [^{14}C]FDP) and A protein. [^{14}C]QA (59,900 dpm) was isolated by the usual procedure and was decarboxylated to nicotinic acid by autoclaving for 2 hrs in glacial acetic acid (10). Carrier nicotinic and quinolinic acids were then added and the mixture was applied to a Dowex-1 formate column. After washing the column with 75 ml of 0.03 N formic acid, nicotinic acid was eluted with 150 ml of

0.3 N formic acid and quinolinic acid with 300 ml of 6 N formic acid. Essentially all of the starting radioacitvity (57,100 dpm) was eluted in a single peak which corresponded exactly to the peak of absorbance of nicotinic acid at 260 nm. Therefore, we are confident that the radioactive compound being measured in these experiments is quinolinic acid.

In previous work on the quinolinate synthetase system of E. coli, we had established that the pH optimum for quinolinate formation was about 8.0 in bicine buffer (2). Subsequently, we found that the E. coli B protein, when assayed alone for its L-aspartate oxidase activity, also has a pH optimum of 8.0 in Bicine buffer (8). It was, therefore, of interest to determine the pH optimum for the reaction catalyzed by the A protein when B protein is replaced by the iminoaspartate-generating system. For the A protein-catalyzed formation of quinolinate from [^{14}C]DHAP and iminoaspartate, the pH optimum in HEPES, the buffer which gives the greatest activity, is 7.0 (Fig.2). Changes in pH would affect the amount of iminoaspartate formed from oxaloacetate and ammonia as well as the catalytic activity of the enzyme. However, since NH_3 should be the species which would react with oxaloacetate to form iminoaspartate and since a higher concentration of NH_3 would be present at pH values greater than 7.0, this probably is the optimum pH for the catalytic activity of A protein. This finding also suggests that the B protein was rate-limiting in the earlier experiment in which the pH optimum of the QA synthetase system was determined to be 8.0 (2).

The finding that high concentrations of OAA and ammonia can replace the aspartic acid and B protein requirement for quinolinate synthesis is not consistent with the claim by others that a) the A protein precedes the B protein reaction in the QA synthetic pathway (12) and b) aspartate undergoes at least 2 transformations before it is condensed with DHAP in the process of QA synthesis (13). It is consistent with our previous findings that the B protein converts aspartate into an unstable intermediate which is

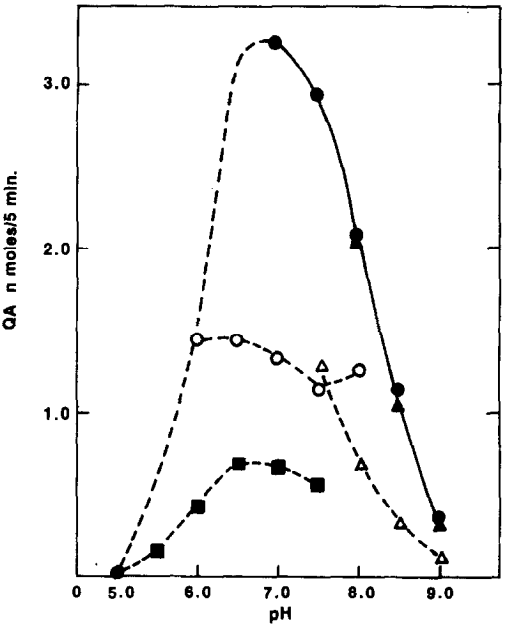
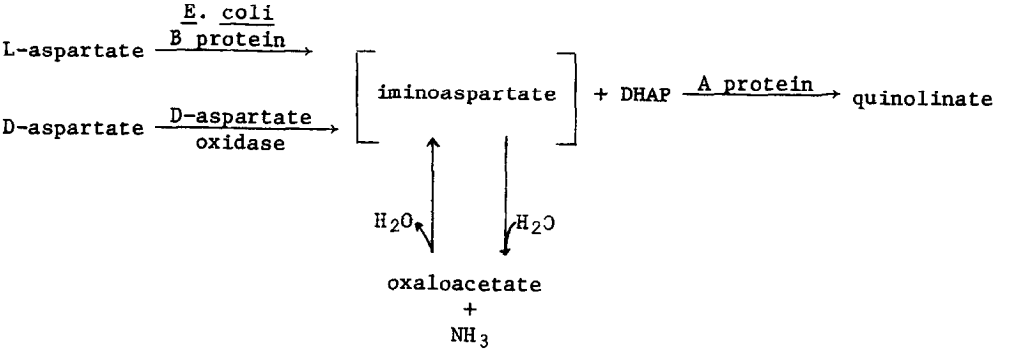


Fig. 2: Formation of [^{14}C]quinolinate from [U-^{14}C]DHAP and iminoaspartate as a function of pH and buffer composition.

The reactions were carried out as shown in Fig. 1 with the exceptions that a different preparation of A protein (1.8 µg per reaction) was used, the source of iminoaspartate was 20 mM (final concentration) oxaloacetate (adjusted to pH 8.0 before addition to the reaction with concentrated ammonium hydroxide) and the buffers indicated were used at a final concentration of 100 mM. ●——●, HEPES; ▲——▲, Bicine; O---O, KPO_4; Δ---Δ, Tris-HCl; ■---■, citrate-K_2HPO_4.

condensed with DHAP by the A protein to form QA (4) and further supports our previous proposals (6,8,14) that QA synthesis in E. coli is a two-step process, which can now be formulated as follows:



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