L-Aspartate Oxidase, a Newly Discovered Enzyme of Escherichia coli, Is the B Protein of Quinolinate Synthetase*

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In Escherichia coli, quinolinic acid, a precursor of NAD+, is synthesized from L-aspartate and dihydroxyacetone phosphate. This synthesis requires two enzymes, a FAD-containing "B protein" and an "A protein." The B protein has been purified 500-fold from E. coli cells. The enzyme behaves as an L-aspartate oxidase. In the absence of A protein, it converts L-aspartate to oxaloacetate. To our knowledge, no enzyme with this activity has been described previously.

The immediate reaction product of the enzyme has the same characteristics (rate of decay to oxaloacetate, condensation with dihydroxyacetone phosphate to form quinolinate) as the unstable reaction product of L-aspartate oxidase. Oxaloacetate is a parabolic function of protein concentration. The L-aspartate oxidase activity also shows marked substrate activation at substrate concentrations above 1.0 mM. The L-aspartate oxidase and B protein activities of the enzyme are inhibited by NAD+, which is competitive with FAD.

We are studying the de novo biosynthesis of pyridine nucleotides in Escherichia coli via QA. In this organism, QA is synthesized from L-aspartate and DHAP. Studies in vitro have led to the purification of two proteins (A and B) required for QA synthesis; the B protein is a FAD-linked flavoprotein (1, 2). Later we found that the E. coli B protein could be replaced by a protein found in mammalian liver (3). However, in a QA synthetase system containing mammalian B protein and E. coli A protein, d-aspartate, but not L-aspartate, was incorporated into QA (4). In other research we have found that this mammalian B protein activity is identical to d-aspartate oxidase, an enzyme which is found in mammalian tissues, especially kidney (5, 6). The unstable compound, iminoaspartate, which is the immediate reaction product of d-aspartate oxidase, is condensed with DHAP to form QA in a reaction catalyzed by E. coli A protein (4). Herein we present evidence that the E. coli B protein is L-aspartate oxidase, an enzyme which, to our knowledge, has not yet been described.

**EXPERIMENTAL PROCEDURES**

Materials—DL-[4-14C]aspartic acid (7.2 mCi/mmol) and L-[U-14C]aspartic acid (200 mCi/mmol) were from ICN. Quinolinic acid, Bicine, HEPES, β-NAD+, FAD, DHAP (Li salt), bovine serum albumin, catalase (beef liver, 2300 units/mg), malate dehydrogenase (pig heart, 945 units/mg), glutamate-oxaloacetate transaminase (pig heart, 95 units/mg), lactate dehydrogenase (rabbit muscle, 600 units/mg), Sephadex G-75, Sephadex G-150, Cibacron-blue, and blue dextran-Sepharose were from Sigma. Dowex ion exchange resins were from Bio-Rad. DEAE-cellulose was obtained from Whatman, and all other reagents were from Fisher.

Growth of Bacteria—The nudB35 (UTH4451) and nadA3 (UTH4673) strains of E. coli K12 were grown and harvested as previously described (7).

Preparation of Pure L- and D-[14C]Aspartic Acids—Pure D-[14C] aspartic acids were prepared from DL-[4-14C]aspartic acid and from L-[U-14C]aspartic acid (which actually contained about 50% of the D-isomer) by the destruction of the L-isomer with glutamate-oxaloacetate transaminase and malate dehydrogenase followed by purification by Dowex-1 column chromatography (4). Pure L-[14C]aspartic acids were prepared by destruction of the D-isomer with purified D-aspartate oxidase, and Dowex-1 chromatography.

Enzyme Assays—QA synthetase B protein and A protein assays were carried out as described previously (1). Three assay methods were used for L-aspartate oxidase. Assay I (2,4-dinitrophenylhydrazine), Assay II (malate dehydrogenase), and Assay III (decarboxylation of 1[14C]oxaloacetate) were carried out as described for the corresponding D-aspartate oxidase assays.

Enzyme Purifications—E. coli A protein was purified from nudB35 cells. E. coli B protein (L-aspartate oxidase) was purified as follows, with all steps carried out at 4°C unless otherwise stated. Frozen I nadA3 cell paste was suspended in 4 volumes of 50 mM KPi, pH 8.0, and sonicated for 15 min with a Bronson Sonifier in a rosette cell immersed in ice water. The sonicated cells were centrifuged for 30 min at 20,000 × g, and the supernatant solution was made 25% in solid ammonium sulfate over 30 min. The precipitate was discarded.

Enzyme Purifications—E. coli A protein was purified from nudB35 cells. E. coli B protein (L-aspartate oxidase) was purified as follows, with all steps carried out at 4°C unless otherwise stated. Frozen I nadA3 cell paste was suspended in 4 volumes of 50 mM KPi, pH 8.0, and sonicated for 15 min with a Bronson Sonifier in a rosette cell immersed in ice water. The sonicated cells were centrifuged for 30 min at 20,000 × g, and the supernatant solution was made 25% in glycerol. This preparation is designated "crude extract" or Step 1. The protein in the crude extract was determined by the 280/290 ratio method. For each milligram of protein, 0.01 ml of 2% proline-sulfate in 50 mM KP was added with stirring over a 30 min period. After stirring for an additional 15 min, the mixture was centrifuged for 15 min at 20,000 × g and the precipitate was discarded.

For Step 2, solid ammonium sulfate was added slowly over 30 min, with stirring, to 50% saturation; stirring was continued for 30 min, and the mixture was centrifuged at 20,000 × g for 15 min. The
precipitate was dissolved in 1/6 the volume of the original crude extract of 5 mM KP, pH 8.0, containing 25% glycerol, and was applied to a Bio-Rad P-6 column (3.5 x 30 cm) which was eluted with the same buffer.

For Step 3, the protein-containing fractions were combined and brought to 35% saturation in sodium citrate by addition of a saturated solution of sodium citrate, pH 8.0. After centrifugation for 15 min at 20,000 x g, the precipitate was dissolved in 16 ml of 5 mM KP, 25% glycerol, pH 8.0, and dialyzed against 2 liters of the same buffer overnight.

For Step 4, any protein which precipitated during dialysis in Step 3 was removed by centrifugation, and the solution was applied to a DEAE-cellulose column (3 x 20 cm) equilibrated with 5 mM KP, 25% glycerol, pH 8.0. The column was then washed with 300 ml of the same buffer containing 0.2 M KCl, and the enzyme was eluted with 400 ml of the same buffer with a linear gradient of 0.2-0.4 M KCl. The activity in the peak was concentrated by precipitation with ammonium sulfate (55% saturation).

For Step 5, the enzyme from Step 4 was dissolved in 2.0 ml of 5 mM KP, 25% glycerol, pH 8.0, and applied to a Sephadex G-150 column (2.5 x 90 cm) eluted and developed with the same buffer.

For Step 6, the active fractions from the Sephadex G-150 column were combined (33 ml) and concentrated to 6.0 ml with a linear gradient of 0.2 M KCl. The column was then washed with 300 ml of the same buffer containing a linear gradient from 0-0.4 M KCl.

Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units x 10^6</td>
<td>units x 10^6/mg</td>
<td>%</td>
</tr>
<tr>
<td>1. Supernatant</td>
<td>30,000</td>
<td>306</td>
<td>8660</td>
<td>1475</td>
<td>0.17</td>
</tr>
<tr>
<td>2. Protamine sulfate supernatant</td>
<td>320</td>
<td>496</td>
<td>1481</td>
<td>9.30</td>
<td>1.8</td>
</tr>
<tr>
<td>3. Sodium citrate</td>
<td>26.5</td>
<td>732</td>
<td>1325</td>
<td>1.83</td>
<td>10.8</td>
</tr>
<tr>
<td>4. DEAE-cellulose chromatography</td>
<td>6.3</td>
<td>138</td>
<td>960</td>
<td>6.10</td>
<td>36</td>
</tr>
<tr>
<td>5. Sephadex G-150 chromatography</td>
<td>33</td>
<td>24.8</td>
<td>923</td>
<td>37.3</td>
<td>219</td>
</tr>
<tr>
<td>6. Blue dextran-Sepharose chromatography</td>
<td>45</td>
<td>4.05</td>
<td>346</td>
<td>85.4</td>
<td>505</td>
</tr>
</tbody>
</table>

Fig. 1. Copurification of E. coli QA synthetase B protein activity and L-aspartate oxidase. A, elution profile of the two activities from a Sephadex G-150 column. Step 5 of enzyme purification as described under "Experimental Procedures." B, elution of the two activities from a blue dextran-Sepharose column. Step 6 of enzyme purification as described under "Experimental Procedures." Dotted line, absorbance at 280 nm; solid circles, QA synthetase activity as measured by the standard assay; open circles, L-aspartate oxidase as measured by assay III (OAA decarboxylation). Both assays are described under "Experimental Procedures." The arrow indicates the beginning of a 0-0.35 M KCl linear gradient.

Table II

Amino acid oxidase activity of purified E. coli B protein

Assays for amino acid oxidase activities were carried out using the three different assay methods described under "Experimental Procedures." In every case, substrate amino acid concentration was 10 mM, and 9.0 µg of B protein, purified through Step 6 (see Table I), was used. FAD was 10 µM in Assay I and 20 µM in Assays II and III except when omitted (-FAD), and oxygen was air-equilibrated buffer except when eliminated by evacuation (-oxygen).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Assay I</th>
<th>Assay II</th>
<th>Assay III</th>
</tr>
</thead>
<tbody>
<tr>
<td>-L-Aspartate</td>
<td>68.4</td>
<td>56.2</td>
<td>65.4</td>
</tr>
<tr>
<td>-FAD</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>-Oxygen</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>-D-Aspartate</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>-L-Alanine</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>-D-Alanine</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

When eliminated by evacuation (-oxygen).

Table III

Requirements for QA and OAA synthesis in the E. coli QA synthetase system

The complete reaction mixture contained A protein (120 µg of Sephadex G-150 fraction), B protein (4.5 µg from Step 6), 20 µM FAD, 0.5 mM L-[4-14C]aspartate (2 mCi/mmol), 0.5 mM DHAP, and 100 mM Bicine (pH 8.0) in a total volume of 0.5 ml. Incubation was carried out at 25°C for 5 min. QA formation and OAA formation (Assay III) were determined as described under "Experimental Procedures.

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>QA formation</th>
<th>OAA formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>4.90</td>
<td>0.18</td>
</tr>
<tr>
<td>-A protein</td>
<td>&lt;0.01</td>
<td>3.88</td>
</tr>
<tr>
<td>-B protein</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>-FAD</td>
<td>0.20</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>-DHAP</td>
<td>&lt;0.01</td>
<td>3.82</td>
</tr>
<tr>
<td>-Oxygen</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
L-Aspartate Oxidase Is the B Protein of QA Synthetase

The aspartate oxidase and QA synthetase activities of the enzyme purified from beef kidney and from E. coli as a function of enzyme concentration. A, D-aspartate oxidase and QA synthetase activities of the FII, Step 5 enzyme purified from beef kidney. B, L-aspartate oxidase and QA synthetase activities of the Step 6 enzyme purified from E. coli. Solid circles, aspartate oxidase activity determined by Assay III (OAA decarboxylation) using 0.5 mM D- or L-[14C]aspartate; open circles, QA synthetase B protein activity using 0.5 mM D- or L-[14C]aspartate in the standard assay for QA formation. Both assays are described under "Experimental Procedures."

FIG. 2. QA synthetase B protein and aspartate oxidase activities of the enzyme purified from beef kidney and from E. coli as a function of enzyme concentration. A, D-aspartate oxidase and QA synthetase activities of the FII, Step 5 enzyme purified from beef kidney. B, L-aspartate oxidase and QA synthetase activities of the Step 6 enzyme purified from E. coli. Solid circles, aspartate oxidase activity determined by Assay III (OAA decarboxylation) using 0.5 mM D- or L-[14C]aspartate; open circles, QA synthetase B protein activity using 0.5 mM D- or L-[14C]aspartate in the standard assay for QA formation. Both assays are described under "Experimental Procedures."

DHAP and A protein are added to the L-aspartate oxidase reaction system (Assay III), L-[14C]aspartate is converted to [14C]QA with only traces of [14C]OAA being formed. However, whenever DHAP or A protein is omitted, no [14C]QA is formed but [14C]OAA is produced. In contrast to the results with the system containing B protein (D-aspartate oxidase) from beef kidney in which equal amounts of QA or OAA were produced,2 the amount of OAA formed when either A protein or DHAP was omitted from the E. coli system was always less than the amount of QA formed when both were present. This phenomenon is due to the nonlinearity of the L-aspartate oxidase activity of the B protein with enzyme concentration. As shown in Fig. 2A, the D-aspartate oxidase and QA synthetase activities of the beef kidney B protein are both linear with enzyme concentration and are quantitatively equal.

In contrast, the QA synthetase activity of E. coli B protein is linear with enzyme concentration while the L-aspartate oxidase activity of this protein is not (Fig. 2B). This nonlinearity is maintained over a wide range of aspartate concentrations, as shown by the data in Fig. 3. The data points for each aspartate concentration closely fitted a line corresponding to the equation [OAA] = k(enzyme)^2/2. The nonlinearity is not influenced by temperature. As shown in Fig. 4, increasing the temperature from 25 to 37°C resulted in an approximate doubling of the rate of the L-aspartate oxidase activity at each enzyme concentration, without affecting the shape of the activity versus enzyme concentration curve. The presence of the A protein fraction had no influence on the nonlinearity of the l-aspartate oxidase activity of the E. coli B protein (data not shown). Addition of boiled B protein (Step 5 or Step 6 enzyme) also had no inhibitory effect on the L-aspartate oxidase activity of E. coli B protein.

The aspartate oxidase and QA synthetase activities of the E. coli B protein are less sensitive to inhibition by dicarboxylic acids and aspartic acid derivatives than the corresponding activities of the beef kidney B protein.2 As shown in Table IV, 10 mM mesotartrate is required for complete inhibition of both activities of the E. coli B protein. In contrast, 10 mM mesotartrate is sufficient to completely inhibit both activities of the beef kidney B protein.6 Even 200 mM mesotartrate did not severely inhibit the A protein-catalyzed condensation of the immediate reaction product of L-aspartate oxidase with DHAP to form QA. This fact allowed us to demonstrate the half-life of this intermediate and to show that QA and OAA are alternate products formed from a common unstable compound, as was done with the D-aspartate oxidase reaction product.2 After a pulse of the intermediate was generated by incubating L-[14C]aspartate with E. coli B protein for 90 s, mesotartrate was added to quench further l-aspartate oxidase activity. The rate of decay of this intermediate was then

<table>
<thead>
<tr>
<th>NaK tartrate concentration (mM)</th>
<th>Product formation (nmol/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>QA: 10.54, OAA: 5.60</td>
</tr>
<tr>
<td>10</td>
<td>QA: 3.16, OAA: 2.42</td>
</tr>
<tr>
<td>100</td>
<td>QA: 0.18, OAA: 0.22</td>
</tr>
<tr>
<td>200</td>
<td>&lt;0.01, &lt;0.01</td>
</tr>
<tr>
<td>400</td>
<td>&lt;0.01, &lt;0.01</td>
</tr>
</tbody>
</table>

DHAP and A protein are added to the L-aspartate oxidase reaction system (Assay III), L-[14C]aspartate is converted to [14C]QA with only traces of [14C]OAA being formed. However, whenever DHAP or A protein is omitted, no [14C]QA is formed but [14C]OAA is produced. In contrast to the results with the system containing B protein (D-aspartate oxidase) from beef kidney in which equal amounts of QA or OAA were produced,2 the amount of OAA formed when either A protein or DHAP was omitted from the E. coli system was always less than the amount of QA formed when both were present. This phenomenon is due to the nonlinearity of the L-aspartate oxidase activity of the B protein with enzyme concentration. As shown in Fig. 2A, the D-aspartate oxidase and QA synthetase activities of the beef kidney B protein are both linear with enzyme concentration and are quantitatively equal.

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E. coli aspartate oxidase activity. The preincubation reaction system contained Step 6 purified E. coli B protein (L-aspartate oxidase), 9 μg; BSA, 0.5%; catalase, 30 units; FAD, 0.02 mM; L-[14C]aspartate 0.5 mM; DHAP, 0.5 mM, and Bicine (pH 8.0), 100 mM in a volume of 0.5 ml. After 90 s of preincubation at 25 °C, 0.5 ml of 1 M NaK mesotartrate was added to stop further E. aspartate oxidase activity. E. coli A protein (Step 5 fraction) was added to duplicate reaction mixtures at the times indicated, and incubation was continued for 5 min. Then the reactions were stopped and QA formation was determined as described under "Experimental Procedures."

The initial reaction mixtures were the same as shown in Fig. 5, with the exception that 3.9 μg of E. coli B protein (Step 6) were used and the initial volume was 0.4 ml. After addition of L-[14C]aspartate (−2 min), the reactions were incubated at 25 °C for 100 s. Then, 0.5 ml of 1.0  M NaK mesotartrate was added (−½ min) to stop further B protein reaction. At 0 min, 60 μg of A protein (Sephadex G-75 fraction) and 0.5 μmol of DHAP (total volume, 0.1 ml) were added and incubations were continued at 25 °C. At the times indicated, reactions were stopped and QA was determined by the standard assay and OAA by Assay III. Any intermediate (iminoaspartate) present was converted to OAA by the acid used to stop the reaction, and therefore "OAA" represents actual OAA plus any remaining iminoaspartate.

<table>
<thead>
<tr>
<th>Time</th>
<th>Operation</th>
<th>&quot;OAA&quot;</th>
<th>QA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Start B protein reaction</td>
<td>0.37</td>
<td>0</td>
<td>0.37</td>
</tr>
<tr>
<td>1</td>
<td>Stop B protein reaction</td>
<td>0.37</td>
<td>0</td>
<td>0.37</td>
</tr>
<tr>
<td>2</td>
<td>Add A protein + DHAP</td>
<td>0.25</td>
<td>0.12</td>
<td>0.37</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.20</td>
<td>0.16</td>
<td>0.36</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.21</td>
<td>0.15</td>
<td>0.36</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.21</td>
<td>0.15</td>
<td>0.36</td>
</tr>
</tbody>
</table>

FIG. 5 (left). Half-life of the unstable intermediate formed by E. coli B protein and converted to QA by E. coli A protein. The pH in various buffers. Standard determinations of L-aspartate oxidase were carried out by Assay III using buffers indicated at a final concentration of 100 mM, L-[14C]aspartate at 0.5 mM with 4.5 μg of Step 6 enzyme and an incubation time of 60 min at 25 °C. □, Bicine; △, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ●, Tris-HCl; ▲, KP; □, Citrate-P.

FIG. 6 (center). L-Aspartate oxidase activity as a function of pH in various buffers. Standard determinations of L-aspartate oxidase were carried out by Assay III using buffers indicated at a final concentration of 100 mM, L-[14C]aspartate at 0.5 mM with 4.5 μg of Step 6 enzyme and an incubation time of 60 min at 25 °C. □, Bicine; △, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ●, Tris-HCl; ▲, KP; □, Citrate-P.

The activity of E. coli L-aspartate oxidase as a function of pH in various buffers is shown in Fig. 6. The pH optimum in Bicine, HEPES, and KP is 8.0. The highest enzyme activity was obtained with Bicine and HEPES, while very low activities were observed with Tris-HCl and citrate-P, and an intermediate activity with KP. The standard assay utilizes Bicine buffer at pH 8.0.

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was varied from 1-20 conditions blue; mM. Cibacron blue acts as a noncompetitive inhibitor, while $K_M$/$3\text{-NAD}^+$ inhibits competitively with FAD.

of apo-L-aspartate oxidase as a function of FAD concentration for enzyme activity (Tables I1 and I11). The apparent in the presence and absence of NAD$^+$ and Cibacron blue. After the blue dextran-Sepharose column purification step, L-aspartate oxidase of animal tissues is highly specific for the $L$-isomer, and the amounts of B protein (or $\alpha$-aspartate oxidase) indicated in this table were used. Aspartate oxidase activity (OAA formation) was determined by Assay III in reaction mixtures lacking A protein. Values in parentheses are theoretical additive values obtained by summing the activities observed with each of the B proteins separately.

<table>
<thead>
<tr>
<th>$[\text{FAD}]_{\mu M}$</th>
<th>$\text{E. coli B protein}$</th>
<th>QA synthetase B protein activity</th>
<th>Aspartate oxidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.73</td>
<td>2.11</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>1.46</td>
<td>3.73</td>
<td>3.81</td>
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<td>1.93</td>
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<td>5.42</td>
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<td>4.02 (3.86)</td>
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<tr>
<td>0.73</td>
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<td>4.75 (4.65)</td>
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</tr>
<tr>
<td>1.46</td>
<td>6.88 (6.42)</td>
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</tr>
<tr>
<td>1.46</td>
<td>8.66 (9.10)</td>
<td>6.61 (6.56)</td>
<td></td>
</tr>
</tbody>
</table>

apparent $K_M$ for L-aspartate is 0.63 mm at substrate concentrations between 0.25 and 1.25 mm, but is 3.33 mm when determined at substrate concentrations of 2.0 to 10.00 mm.

Fig. 8 shows a Lineweaver-Burk plot of the initial velocity of apo-$\alpha$-aspartate oxidase as a function of FAD concentration in the presence and absence of NAD$^+$ and Cibacron blue. After the blue dextran-Sepharose column purification step, E. coli L-aspartate oxidase is completely dependent on added FAD for enzyme activity (Tables I1 and I11). The apparent $K_M$ for FAD, calculated from the data shown in Fig. 8, is 2.5 mm. Cibacron blue acts as a noncompetitive inhibitor, while $\beta$-NAD$^+$ inhibits competitively with FAD.

**DISCUSSION**

The results presented here strongly suggest that the B protein of the E. coli QA synthetase system is a FAD-linked $\alpha$-aspartate oxidase. To our knowledge, no $\alpha$-aspartate oxidase has yet been described. It is not included in the latest compilation of enzymes (8). L-aspartate is oxidized very slowly by snake venom L-amino acid oxidase (9), and not at all by the mammalian B protein (10). The intensively-studied $\alpha$-aspartate oxidase of animal tissues is highly specific for the $L$-isomer (5, 11). The same intermediate product (iminoaspartate) would be expected to be formed by both $\alpha$- and L-aspartate oxidases. Our experimental evidence supports this concept, since the immediate products of both enzymes decay spontaneously to form OAA with the same rate, at a given temperature and pH. More importantly, the immediate reaction product of both enzymes is converted to QA when DHAP and the A protein of the QA synthetase system are present.

Mammalian $\alpha$-aspartate oxidase and E. coli L-aspartate oxidase do, however, differ markedly in one respect. When E. coli L-aspartate oxidase is assayed in its B protein role by the formation of QA, enzyme activity is linear with protein concentration over a rather wide range. However, when assayed for L-aspartate oxidase activity, this activity is proportional to the square root of the enzyme concentration (Fig. 2B). This nonlinear relationship between L-aspartate oxidase activity and enzyme concentration is maintained over a wide range of substrate concentrations (Fig. 3) and temperatures (Fig. 4). This behavior is not due to product(s) inhibition (Table VI), and is not abolished by the addition of either A protein or DHAP alone. When both A protein and DHAP are present, a linear relationship between L-aspartate oxidase concentration and enzyme activity is restored (enzyme activity is stimulated); under these conditions QA is the product formed, rather than OAA. A model which is consistent with these observations is as follows.

$$\text{B} \cdot \text{B} \rightarrow 2\text{B L-aspartate oxidase}$$

inactive dimer (inactive dimer)$$

$2(\text{A} \cdot \text{DHAP}) \rightarrow 2(\text{B} \cdot \text{A} \cdot \text{DHAP})$ QA synthetase

**Scheme I**

In this model, B protein associates into an inactive dimer, but A protein, in the presence of DHAP, prevents this association. However, a complex formation between A and B as shown above is certainly not necessary for QA synthesis, since a heterologous protein, mammalian $\alpha$-aspartate oxidase, can replace the E. coli B protein. QA synthesis occurs if A and B proteins are physically separated by a dialysis membrane (7), and the B protein requirement can be replaced altogether by chemically generated iminoaspartate. 3

The nonlinear behavior of the L-aspartate oxidase activity of E. coli B protein might serve a regulatory function, since it would greatly reduce the conversion of L-aspartate to OAA by this enzyme under conditions when QA was not being synthesized (absence of active A protein or of available DHAP).

A Lineweaver-Burk plot of the L-aspartate oxidase activity of E. coli B protein as a function of L-aspartate concentration (Fig. 7) gives an indication of substrate activation at concentrations of aspartate above 1.0 mm. This type of kinetic behavior is not without precedent, (e.g. Ref. 12), and may indicate that the substrate is capable of occupying an allosteric activator site on the enzyme, as well as the normal catalytic site.

The L-aspartate oxidase activity of E. coli B protein varies greatly with buffer and pH (Fig. 6). Phosphate is definitely inhibitory, while Bicine and HEPES give the best activity of those buffers tested. The pH optimum of 8.0 for L-aspartate oxidase activity is the same as that previously established for QA synthetase in a crude system (1). In this earlier work, B protein was probably the rate-limiting step in the QA synthetase system, since the A protein-catalyzed reaction appears to have a pH optimum of about 7.0. 

NAD$^+$ is a competitive inhibitor of L-aspartate oxidase with respect to FAD (Fig. 8). This finding explains the rather variable NAD$^+$ inhibition of preparations of QA synthetase which differed in the presence or absence of added FAD (1, 2, 13).

The formation of QA from aspartate and DHAP requires 2 condensation reactions and the removal of inorganic phosphate, 2 hydrogens, and 2 water molecules (14). This led to the conclusion that several intermediate compounds should be involved in QA synthesis (14). Experimental results have been published (14) and interpreted as evidence for the formation of several stable intermediates between aspartate and QA. However, all our findings are consistent with only two enzymes and one free, but unstable, intermediate. Scheme II presents a hypothetical reaction mechanism by which all the chemical transformations involved in the conversion of DHAP and iminoaspartate to QA could be carried out on the surface of the A protein. Electron-withdrawing groups in iminoaspartate would facilitate removal of a proton from carbon 3 (Reaction A). The species thus formed, having partial carbanion character, could then carry out a nucleophilic attack on carbon 3 of DHAP with the elimination of inorganic phosphate (Reaction B). The condensation product thus formed could then undergo a keto-aldol isomerization analogous to the triose phosphate isomerase-catalyzed reaction (Reactions C and D). Loss of a proton from the resulting molecule (Reaction E) would produce an amino aldehyde which would undergo a Schiff-base formation (Reaction F) followed by a dehydration (Reaction G) to produce QA. The overall reaction sequence would have a high $K_m$ due to the very large negative free energy change involved in aromatic ring formation. This may appear to be a large number of reactions to be catalyzed by a single enzyme of 35,000 daltons (1). However, almost as many steps are required for the synthesis of 2,3-dihydroxycarnitonic acid, which is formed from pyruvate and aspartate semialdehyde, apparently by a single condensing enzyme (15). Also, Reactions F and G would almost certainly occur spontaneously in the absence of any enzyme catalysis.

The scheme presented above differs radically from the pathway proposed by Tritz and co-workers (16): Aspartic acid $\rightarrow$ $x$ $\rightarrow$ $y$ + DHAP $\rightarrow$ z $\rightarrow$ QA. In this formulation, z is thought to represent several intermediates (14, 16). This proposed pathway differs from ours in that (a) the order of the A and B protein-catalyzed steps is reversed and (b) three, or perhaps five (14), stable intermediates are postulated, rather than one unstable intermediate. Kerr and Tritz (17) deduced that the A protein-catalyzed step proceeds the B protein-catalyzed reaction from the results of a cross-feeding experiment. When a nadA and a nadB mutant, each with distinctive side markers, were mixed together in a niacin-free medium, the nadA mutant continued to grow while the nadB did not, suggesting that the nadB mutant was excreting a compound that fed nadA and, therefore, that A occurs before B in the QA synthetic pathway. An alternate interpretation of these results may be provided by the study of Lundquist and Olivera (18) on niacin starvation of E. coli. They found that when cells of a niacin-requiring mutant of E. coli are deprived of niacin, they continue to divide for 2 h. Then division stops,
L-Aspartate Oxidase Is the B Protein of QA Synthetase

and during late starvation leakage of the intracellular pyridine nucleotides into the medium occurs. About 60% of this pool is excreted in 4 h, apparently in the form of nicotinic acid riboside, although the cells remain intact and viable (18). If the two E. coli strains used in the cross-feeding experiment differed in their susceptibility to niacin starvation, and the nadvB mutant could have begun to excrete nicotinic acid riboside which was utilized by the nadA mutant, then the results observed could be explained. Since no evidence was presented that the total concentration of pyridine nucleotides in the mixed culture increased with cell number (17), and since growth of E. coli can continue when the pyridine nucleotide content is only 2% of normal (18), this alternate explanation cannot be excluded. The isolation of a compound which supports the growth of both a nadvA and a nadvB mutant from the culture fluid of a niacin-starved nadvC mutant (19), which cannot metabolize QA, could also be due to the excretion of nicotinic acid riboside by this latter mutant. Our evidence for the placement of B before A in the pathway is based upon (a) the observation that the B protein-reaction product was converted to QA when centrifuged through an Amicon filter cone into a solution containing A protein, but not vice versa (7), (b) the enzymatic evidence presented in this paper and in our unpublished results, and (c) the finding that only A protein plus DHAP is required for QA synthesis when iminoaspartate is chemically generated.3

The conclusion that multiple, stable intermediates occur in the pathway of QA synthesis is based on the observation that [14C]-labeled aspartate and/or fructose 1,6-bisphosphate were converted, by incubation with a crude extract of an E. coli nadvC mutant, into six [14C]-labeled compounds which were separated by paper chromatography (14). Since formation of these [14C]-labeled compounds was repressed by nicotinic acid (13), aspartate and/or fructose 1,6-bisphosphate were not converted to QA "at a more rapid rate than the synthesis of QA from aspartate," the data presented do not rigorously support this conclusion (16), and it is possible that the isolated compound was being converted to QA via aspartate in the crude system used.

Since we have not yet been able to purify the A protein to homogeneity due to its lability, it is still possible that another enzyme, or enzymes, may be present in this preparation, and that free intermediates subsequent to the condensation of DHAP and iminoaspartate may occur. However, no evidence consistent with this concept has been found during our series of in vitro studies, and no convincing evidence to support this idea has been educed by others. Furthermore, the picture of the QA synthetic pathway which has emerged from our enzyme studies is quite consistent with the previous genetic analysis and in vitro complementation studies (21), which indicated that only two enzymatic systems and one intermediate are involved in QA synthesis from aspartate and DHAP.

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REFERENCES
L-Aspartate oxidase, a newly discovered enzyme of Escherichia coli, is the B protein of quinolinate synthetase.
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