Occurrence in Mammalian Liver of a Protein which Replaces the B Protein of E. coli Quinolinate Synthetase*,+

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SUMMARY

Escherichia coli contains two proteins (A and B) which together convert dihydroxyacetone phosphate and aspartate to quinolinic acid, a precursor of NAD. Although mammalian liver homogenate does not catalyze this reaction it contains a protein which will replace the B protein of the E. coli system. The behavior of the liver protein on Sephadex G-75 suggests it is much smaller than the E. coli B protein. Liver B protein also appears to contain tightly bound FAD while FAD is easily removed from the E. coli B protein. The pH optimum for the hybrid system E. coli A protein-liver B protein is 9.0 while in the pure E. coli system the optimum is pH 8.0. The hybrid system is inhibited by NAD to the same extent as the pure E. coli system.

INTRODUCTION

The conversion of tryptophan to niacin in rats was established in 1945 (1), and $QA^{\dagger}$ was shown to be a metabolite of tryptophan in mammals a few years later (2). QA was firmly established as an intermediate in the conversion of tryptophan to NAD in mammals by the discovery of an hepatic enzyme forming nicotinic acid mononucleotide from QA (3, 4). However, tryptophan was shown not to be a precursor of niacin in E. coli or B. subtilis (5) or in higher plants (6) and studies with labeled precursors indicated that succinate and glycerol are incorporated into the pyridine ring of niacin in E. coli (7). More recently studies in our laboratory have shown that in E. coli QA is synthesized from DHAP and aspartate (8, 9). QA synthesis in E. coli also requires FAD and is catalyzed by two proteins which we have designated A and B (8). No evidence for the formation of a stable intermediate by either of

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†Abbreviations used QA-quinolinic acid DHAP-dihydroacetone phosphate.

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these proteins alone has been obtained in our laboratory, although studies of products excreted by E. coli mutants suggest the occurrence of one or more intermediates between aspartate and QA (8, 9). QA is also converted to nicotinic acid mononucleotide by an enzyme present in E. coli (12). Thus QA is a precursor of the pyridine nucleotides in both mammals and E. coli, although its origin in these diverse organisms is quite different.

We wish to report the surprising discovery that mammalian liver contains a protein which will fully replace the B protein of the E. coli QA synthetase system. This protein will not catalyze the formation of QA in the absence of the E. coli A protein, and only traces of A protein activity can be detected in mammalian liver.

EXPERIMENTAL METHODS

Materials. [U^{14}C]L-aspartic acid was purchased from New England Nuclear and N, N-bis-2-hydroxyethylglycine (Bicine) from Calbiochem. Quinolinic acid, dihydorxyacetone phosphate (dimethyl ketal) FAD, NAD, NADH, NADP, NADPH were obtained from Sigma. The ketal was hydrolyzed immediately before use according to the manufacturer's directions. Dowex resins and DEAE-cellulose were purchased from Bio-Rad. Sephadex G-75 was from Pharmacia.

Methods. Growth of bacteria. A nadB mutant of E. coli K-12 (SB-16) was grown for 18 h with vigorous aeration at 37C on the minimal medium of Yates and Pardee (13) supplemented with 10^{-3}M methionine, 2 x 10^{-6}M thiamine and 5 x 10^{-7}M nicotinic acid. The bacteria were harvested with a Sharples centrifuge, washed and stored at -15C.

Assay Method. The reaction mixture contained 0.25 µmole of [U-^{14}C]L-aspartic acid (spec. act. 4 Ci/mole) 0.3-0.4 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 25C for 15 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 (14). After measuring the absorbance at 268 nm the radioactivity was determined by counting in Brays solution with a Beckman scintillation spectrometer and the yield of quinolic acid calculated.

Protein concentration was determined by the method of Warburg and Christian (15).

The E. coli A protein used in these experiments was purified by a modification of the methods previously described (8) but with a considerably improved enrichment and final specific activity. The steps were a) 2% protamine sulfate precipitation, b) 40-60% saturated ammonium sulfate, c) DEAE-chromatography with a 0.1M-0.4M KCl gradient, d) Sephadex G-75 chromatography. The final enrichment of A protein is 120 to 150 fold over the crude extract. Details of this purification will be presented elsewhere.

Table 1

Presence of B Protein Activity in Liver Homogenate

ENZYME SOURCE	ADDITION	UNITS QA SYNTHETASE
18,000 x g Supernatant calf liver homogenate	none	0.01
calf liver homogenate	B protein (E. coli)	0.05
calf liver homogenate	A protein (E. coli)	0.43
Dialized supernatant calf liver homogenate	none	0.02
calf liver homogenate	B protein (E. coli)	0.06
calf liver homogenate	A protein (E. coli)	0.82

Calf liver was finely ground by passing 3 times through a hand operated meat grinder and then vigorously stirred for 10 min at 4C with 4 volumes of 0.05M KPO_4 pH 8.0 containing 0.05M KCl. The homogenate was centrifuged for 30 min at 18,000 x g. Glycerol was added to the supernatant to 25% final concentration. A 5 ml aliquot of this supernatant was dialized for 16 hrs at 4C against 2 liters of the homogenization solution containing 25% glycerol. Protein concentration (280/260) before dialysis 33.4 mg/ml after dialysis 31.1 mg/ml. Assays were carried out in duplicate using the standard system described in Methods with 0.1 ml of E. coli B protein Sephadex fraction (26 µg protein) or 0.1 ml of E. coli A protein Sephadex fraction (60 µg protein) and 0.05 ml of calf liver homogenate. Units nmoles QA/mg protein of calf liver homogenate/15 min.

RESULTS AND DISCUSSION

Hepatic B protein activity was first detected serendipitously in rat liver homogenate during a search for factors which might stabilize the A protein of the E. coli QA synthetase system. The experiments reported here were carried out with calf liver because of the ease of obtaining this tissue. As shown in Table I a 18,000 x g supernatant of calf liver homogenate did not catalyze an appreciable synthesis of QA from DHAP and aspartate and showed only traces of A protein activity. However, when a purified preparation of E. coli A protein was added to the liver homogenate QA was synthesized, indicating the presence of B protein activity. This activity was signifi-

cantly increased following dialysis. The specific activity of the B protein in the crude dialized homogenate under these assay conditions is of the same order of magnitude as that observed in crude extracts of E. coli nadA mutants (1-1.5 units/mg protein). When assay conditions were optimized for the liver B protein its specific activity was found to be actually higher than that of E. coli (5-6 units/mg protein in crude extracts). Boiled liver homogenate had no activity with the E. coli A protein and all of the liver B protein was precipitated by ammonium sulfate at 55% saturation.

Although our two column assay procedure is highly specific for the isolation of QA (14), the finding of B protein activity in liver was so unexpected that we carried out an experiment to establish independently that our measured reaction product was actually QA, and to eliminate the possibility that the E. coli A protein and liver homogenate were together catalyzing the conversion of DHAP and [^{14}C]-aspartate into a compound not resolved from QA in our assay. A 4 fold reaction was carried out using a 0-50% ammonium sulfate fraction of liver homogenate as the B protein source. The QA fraction was isolated on our usual analytical columns and decarboxylated to form nicotinic acid as described by Henderson and Hirsch (2). When the decarboxylation produced was chromatographed on a Dowex-1-formate column (Figure I) only one peak of 260 nm absorbance and radioactivity appeared corresponding to the elution position for nicotinic acid. This peak contained 73% of the total radioactivity of the QA fraction from our second assay column. Since ¼ the ^{14}C in QA formed from [U-^{14}C]-aspartate is lost on decarboxylation to nicotinic acid, this represents almost quantitative recovery of our QA fraction as nicotinic acid and establishes the reaction product of the mixed E. coli-liver systems as QA.

We then subjected the ammonium sulfate fraction from liver to Sephadex G-75 chromatography on the same column used in purification of the E. coli A protein. The elution pattern of liver B activity is shown in Figure 2. The liver B protein elutes at almost the same position as the E. coli A protein

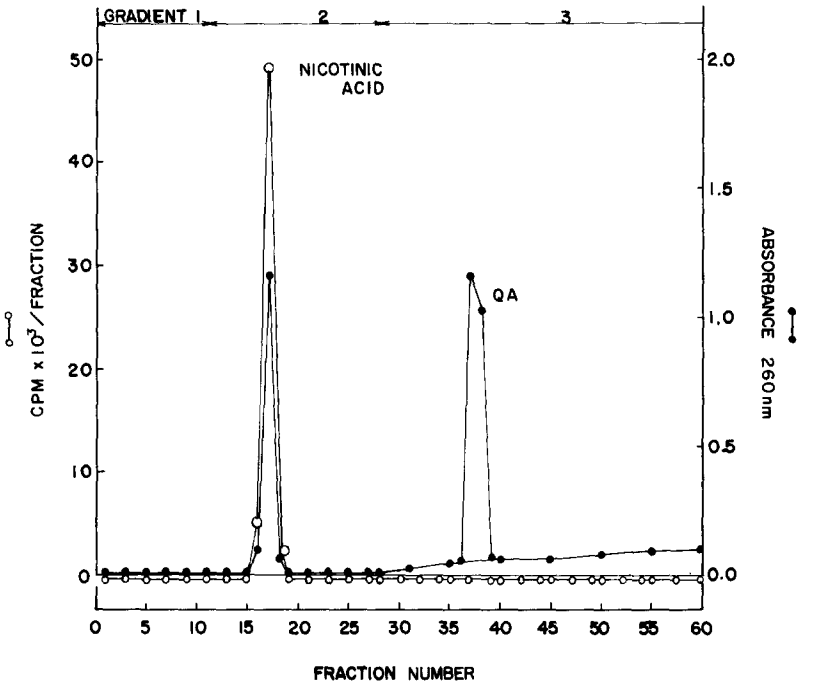

Fig. 1: Chromatography of the decarboxylated reaction product (nicotinic acid)

A 4 fold reaction mixture containing the usual concentrations of reactants with 0.4 ml of E. coli A protein Sephadex fraction (240 µg protein and 0.2 ml of liver 0-50% ammonium sulfate fraction (16 mg protein) in a total volume of 2 ml was incubated and deproteinized under the usual conditions. 2.0 ml of 2.0 mM QA was added and the QA fraction isolated in the usual assay columns. An aliquot of the QA fraction was counted (total 78,000 cpm) and the remainder taken to dryness on a rotary evaporator. The residue was carefully dissolved in 5 ml of glacial acetic acid and autoclaved for 2 hr at 15-20 lbs pressure in a sealed glass tube. The acetic acid was removed by evaporation. The residue was dissolved in 5 ml H_2O, the pH was adjusted to 7.0 with KOH and the solution was applied to a Dowex 1 x 2 formate column (1 x 30 cm). The column was developed with a stepwise gradients of formic acid and 10 ml fractions were collected. Absorbance of 260 n meters was determined on each fraction and an aliquot was counted by liquid scintillation. Total cpm in nicotinic acid fraction 56,600. ●—● Absorbance 260 n meters; o——o cpm ^{14}C. Position of QA determined in a separate column run. Gradient I - linear gradient of 75 ml of 0.03 N formate into a mixing vessel containing 150 ml H_2O (75 ml collected). Gradient II - 0.03 N formated replaced by 150 ml of 0.3 N formate (150 ml collected). Gradient II - 0.3 N formate replaced by 300 ml of 6.0 N formate.

which has an apparent molecular weight of 35,000 (8). In contrast the B protein from E. coli has an apparent molecular weight of 85,000 (8).

The requirements for QA synthesis using the Sephadex fractions of E. coli A and B proteins and of E. coli A protein and liver B protein are shown in

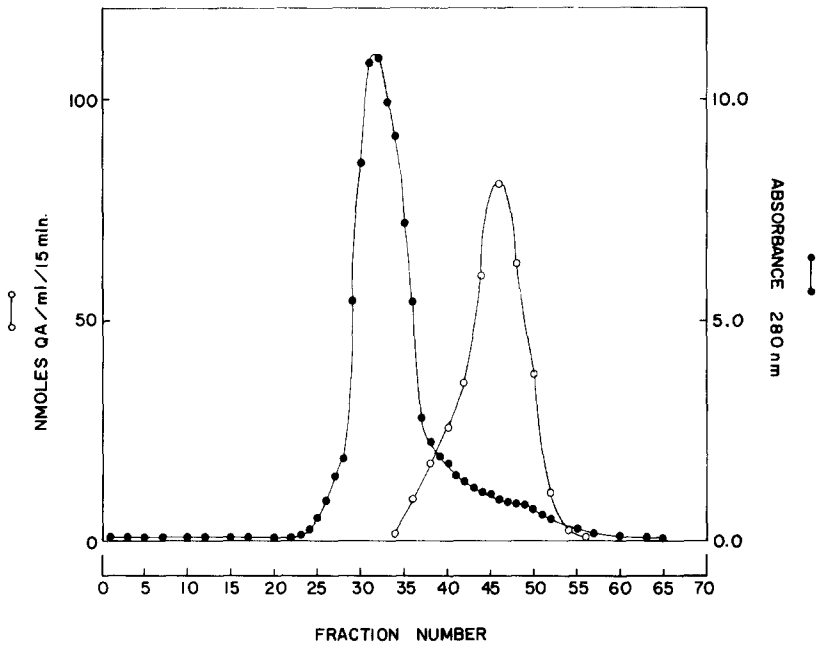

Fig. 2: Elution of calf liver protein B activity from a Sephadex G-75 column.

A homogenate was prepared as described in Table I from twenty grams of calf liver with 40 ml of 0.25 M sucrose 0.05 M potassium PO_4 pH 8.0. The homogenate was centrifuged for 30 min at 18,000 x g and a 30-55% saturation ammonium sulfate fraction prepared from the supernatant solution. This ammonium sulfate precipitate was dissolved in 2.8 ml of 0.005 M potassium phosphate pH 8.0 containing 0.05 M KCl and 25% glycerol (buffer A) and dialized 16 hrs at 4C against 2 liters of the same buffer. A slight precipitate was removed by centrifugation at 16,000 x g for 15 min and the supernatant solution (5 ml) was applied to a 2.6 x 85 cm Sephadex G-75 column which was equilibrated and eluted with buffer A. Fractions of 5 ml were collected and assayed for absorbance at 280 n meters and B protein acitivity. ●—● A280, o—o B protein activity (nmole QA/15 min/ml) in the standard assay.

Table II. The E. coli system requires added FAD for activity while the mixed system using liver B protein does not. This suggests that B is a flavoprotein, and that FAD binding is much tighter to mammalian B than to E. coli B.

Another marked difference between the hybrid QA synthetase system of E. coli A protein and liver B protein and the all E. coli system is the optimum pH for QA formation. The E. coli system has an optimum between pH 8.0 and pH 8.5 (8), while the hybrid system has a sharp optimum peaking at pH 9.0. (Data not shown). The hybrid liver-E. coli synthetase system is inhibited

Table 2

Requirements for QA synthesis in the E. coli system and the hybrid E. coli-calf liver system.

Reaction System	QA nmoles/15 min
Complete E. coli system	3.28
— protein A	<.01
— protein B	<.01
— DHAP	.07
— FAD	.04
Complete E. coli-liver system	3.89
— protein A (from E. coli)	<.01
— protein B (from calf liver)	<.01
— DHAP	0.58
— FAD	3.86

The standard assay system was used with 60 µg of E. coli A protein Sephadex fraction and 26 µg of E. coli B protein Sephadex or 102 µg of calf liver B protein Sephadex fraction. Values are average of duplicate assays.

by NAD^+ to about the same extent as the all E. coli system. NAD^+ at 1.0 mM produced a 57% inhibition of the hybrid system as compared to a 54% inhibition previously reported in the all E. coli system (9). The results of a partial purification of the B protein from calf liver are shown in Table III. The specific activity of the B protein in calf liver homogenate is higher than that previously observed in any E. coli mutant crude extract (9) and this tissue would appear to be an excellent source for purification of this protein.

The presence in liver of a protein which can replace with high activity the B protein of the E. coli quinolinate synthetase system is very puzzling. Several possible explanations suggest themselves. 1) Mammalian liver contains the DHAP-aspartate pathway of QA synthesis in addition to the well established tryptophan pathway, but the A protein component is so labile that

Table 3

Partial purification of the B protein from calf liver

STEP	Total Activity (units)	Total Protein	Specific Activity units/mg protein	Purification Fold	Yield %
Dialized homogenate	6,520	1,190	5.5	1.0	100
30-55% Saturated $(NH_4)_2SO_4$	6,040	520	11.6	2.1	93
Sephadex G-75 pooled fractions	5,338	72.6	73.5	13.4	82

The purification scheme is described in the legend of Figure 2. QA synthesis was assayed in the standard reaction mixture with 370 µmoles of DHAP per assay and Bicine pH 9.0 rather than 8.0. E. coli A protein Sephadex fraction 60 µg per assay. Units are nmoles of QA synthesized in 15 min.

we cannot detect its activity. This seems unlikely in view of nutritional data which shows that mammals require either niacin or tryptophan, although the DHAP-aspartate pathway could be present, but at a level insufficient to meet the needs for pyridine nucleotide biosynthesis. 2) The A protein has been lost but the B protein has been retained as an evolutionary vestige which now has no function in liver. 3) The B protein catalyzes the formation of an intermediate which is the precursor of QA (via the A protein reaction) in E. coli but of another product in liver. 4) The B protein alone has a function other than QA synthesis in both E. coli and liver. The A protein is a modifier which converts the B protein into quinolinate synthetase. This situation would be analogous to lactose synthetase in which the A protein is a widely distributed UDP galactosyl transferase which is converted to lactose synthetase by binding of the B protein (α-lactalbumin) in the mammary gland (16).

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