ApbA, the Ketopantoate Reductase Enzyme of Salmonella typhimurium Is Required for the Synthesis of Thiamine via the Alternative Pyrimidine Biosynthetic Pathway*

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The apbA gene of Salmonella typhimurium was shown to encode ketopantoate reductase. ApbA was purified from crude cell-free extracts to greater than 95% homogeneity after two chromatographic steps. N-terminal amino acid sequencing (first 15 amino acids) and Western blot analysis confirmed the isolated protein was ApbA. The functional protein was a monomer with a molecular mass of 31.1 kDa. Optimal reaction conditions for the reduction of ketopantoic acid were established at a pH of 6.25, and a temperature of 42 °C. The preferred electron source was NADPH, and the apparent Km constants of the enzyme for NADPH and ketopantoic acid were determined to be 0.776 ± 0.09 mM and 0.742 ± 0.01 mM, respectively. The homogeneous enzyme had a specific activity of 64.3.

The alternative pyrimidine biosynthetic (APB)1 pathway allows synthesis of thiamine in Salmonella typhimurium in the absence of de novo purine biosynthesis (1, 2). The apbA gene was the first genetic locus found to be required for function of the APB pathway (3). Computer analysis of the apbA sequence identified a putative NAD/FAD binding site suggesting ApbA was an oxidoreductase enzyme. Recent efforts to define the in vivo function for ApbA were led by the observation that pantothenate, as well as thiamine, could correct the nutritional requirement of an apbA mutant.2 Further experiments indicated that under conditions where apbA mutants required thiamine, the pantoic acid moiety of pantothenate was sufficient to restore growth.2

Because of the putative oxidoreductase activity of ApbA, we focused on the step in pantoic acid synthesis that involved the reduction of ketopantoic acid (KPA) (Fig. 1). Interestingly, acetohydroxy acid isomeroreductase (iIvC), an enzyme required for branched chain amino acid biosynthesis, has been shown to catalyze this reduction in crude cell-free extracts (5). Residual KPA reductase activity measured in an ilvC mutant was attributed to the KPA reductase encoded by the panE gene (5). Genetic data presented elsewhere shows that contrary to earlier mapping data, apbA is the locus previously designated panE.2 The symbol of apbA has been retained to reflect the involvement of this enzyme in thiamine biosynthesis.

KPA reductase activities have been identified and characterized to different extents in Saccharomyces cerevisiae, Escherichia coli, and Pseudomonas maltophilia 845 (6, 7). This work describes the completed purification of the KPA reductase from S. typhimurium encoded by the apbA gene. KPA reductase has a well described role in pantothenate biosynthesis in S. typhimurium and E. coli. However, the previously described phenotypes of an apbA mutant (3) suggests that there is also a role for ApbA in thiamine synthesis via the APB pathway.

EXPERIMENTAL PROCEDURES

Materials

Culture media supplies were obtained from Difco, and growth of bacterial cultures was monitored using a Manostat Klett colorimeter with a red filter (Manostat Corp., NY). Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was obtained from Fisher. Ketosovalerate, NADPH, NADH, and ammonium sulfate were obtained from Fluka (Milwaukee, WI), and Bakerbond Wide Pore Hi-propyl (C₃) resin was obtained from J. T. Baker (Phillipsburg, NJ). Q-Sepharose Fast Flow and all other chemicals were obtained from Sigma. Restriction enzymes were from Promega (Madison, WI), oligonucleotides were from Genosys Inc. (The Woodlands, TX), and Pfu recombinant DNA polymerase was from Stratagene (La Jolla, CA). Plasmid vector pET-14b was obtained from Novagen (Madison, WI).

Ketopantoyl lactone was prepared as described (8), and stock solutions were stored in 15 mM HCl due to the instability of this compound at pH >2. KPA was prepared as described elsewhere (6).

Molecular Biological Techniques

E. coli strain BL21/DE3 was obtained from Novagen, Inc. Plasmids pT7–5 and pT7–6 and methods for overexpressing proteins with this system have been described (9). Plasmid pT7–apbA was constructed by cloning a BamHI–HindIII fragment containing the promotorless apbA coding sequence into the multiple cloning site of plasmid pT7–5 (3). The construction was confirmed by sequence analysis, and the resulting plasmid was transformed into the overexpression strain BL21/DE3, which carried in its genome the gene encoding T7 RNA polymerase under the control of an IPTG inducible promoter.

A construct containing an N-terminal His₆-ApbA fusion protein was generated by engineering an NdeI restriction site immediately 5′ of the translation initiation codon of apbA followed by ligation of the necessary fragment into an appropriate vector as described by the manufacturer. The resulting construct was confirmed by sequence analysis.

Overexpression Conditions

In a standard overexpression experiment, 4 liters of BL21/DE3 cells containing pT7–apbA were grown in LB medium containing 50 µg/ml carbenicillin at 37 °C to an OD₆₀₀ of 0.6. IPTG was added to a final concentration of 0.4 mM to induce expression of the T7 RNA polymerase. After 3 h of growth, cells were centrifuged, yielding a cell pellet of approximately 15 g, which was resuspended in 60 ml of 25 mM potassium phosphate buffer, pH 7.5. Cell-free extracts were generated by sonicating cells on ice for four 30-s bursts at 50% duty cycle with a Fisher Model 550 Sonic Dismembrator. Cell-free extracts were clarified by centrifugation at 48,000 × g for 1 h at 4 °C in a Beckman J2-HS centrifuge.
**Protein Quantitation and Manipulation**

**Quantitation**—A Pierce bicinchoninic acid (BCA) or Micro BCA Protein assay reagent kit was used to quantify protein. Ultrapure bovine serum albumin was used to generate a standard curve. Standard buffer (25 mM potassium phosphate buffer, pH 7.5) was used unless otherwise specified.

**His$_6$-ApbA**—After induction, His$_6$-ApbA was purified to >95% homogeneity using an Ni$_2$$^+$/NTA affinity column developed under denaturing conditions in the presence of 6 M guanidine HCl. Rabbit polyclonal antibodies against His$_6$-ApbA were generated at the animal care unit of the University of Wisconsin Medical School. Antisera was prepared and titered according to Harlow and Lane (10).

**SDS-Polyacrylamide Gel Electrophoresis (PAGE)**—PAGE was performed by the method of Laemmli (11), and protein bands were visualized after staining with either (a) 40:55:5:0.05 ethanol:water:acetic acid:Coomassie R250 and destained in 40:55:5: ethanol:water:acetic acid; or (b) 1 × SYPRO Red in 7.5% acetic acid. Gels stained in SYPRO Red were visualized on a standard Fisher 312 nm transilluminator. Percent of total protein was estimated by densitometric scans using a GS300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA).

**Western Analysis**—SDS-PAGE was performed as described above. Western analysis was performed as described by Harlow and Lane (10). Alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) was used to detect anti-ApbA bound to the membrane support, as described (10).

**N-terminal Amino Acid Sequencing**—Electroblotting of SDS-PAGE gels onto polyvinylidene difluoride membranes (Pierce) was done as follows. A 12.5% SDS-PAGE gel containing samples of purified ApbA was soaked in 10 mm CAPS, pH 11.0, 10% MeOH for 20 min, and loaded into a Bio-Rad Mini Trans Blot cell. Transfer was run at 4 °C at 100 V for 1 h. Amino acid sequence analysis was performed at the Protein and Nucleic Acid Shared Facility of MCW Research Services, Medical College of Wisconsin.

**Molecular Mass Determinations**—The molecular mass of native ApbA was determined by HPLC gel filtration using a BioSep-SEC-S2000 column (300 × 7.8 mm) (Phenomenex; Torrence, CA). The column was equilibrated and run in 50 mM potassium phosphate buffer, pH 7.5. Flow rate was set at 1.0 ml/min on a Waters 600 HPLC system (Millipore Corp., Milford, MA). Protein elution from the column was monitored at 280 nm by a Waters 996 Photodiode Array detector. Standard molecular weights were calculated using Bio-Rad gel filtration standards (670-kDa thyroglobulin; 158-kDa bovine gamma globulin; 44-kDa chicken ovalbumin; 17-kDa equine myoglobin).

**Biological Activity Assays**—To test biological activity of reaction components, dried paper chromatograms on which reaction mixtures had been run as described below were cut into 1-cm cross-sections and placed individually in microcentrifuge tubes with 100 μl of double-distilled water (ddH$_2$O). After incubating for 30 min, the paper was removed, placed in a cellulose acetate filter insert (Costar, Cambridge, MA), and centrifuged at 12,000 × $g$ for 60 s in a Maraton 13KMM (Fisher, Itasca, IL). Eluant was dried to completion on a Savant Speed Vac concentrator and resuspended in 10 μl of ddH$_2$O. Fractions were assayed for biological activity on minimal glaconate plates overlaid with soft agar (0.7% w/v) in which cells of strain DM377 (panB) were suspended. A positive control was afforded by commercial pantoyl-lactone.

**In Vitro KPA Reductase Assay**—KPA reductase activity during ApbA purification was assayed as described (6). The reaction mixture contained KPA (2.5 μmol), potassium phosphate buffer, pH 7.5 (200 μmol), and enzyme (~0.05 nmol) in a total volume of 2.0 ml. Reaction mixtures were incubated at 42 °C for 2 min prior to addition of NADPH (0.5 μmol) to initiate the reaction. Future experiments with homogenous protein employed 3.1 μmol of KPA and 3.1 μmol of NADPH in a total volume of 1 ml. A typical assay was followed for 2 min, with the initial rate determined within 30 s. The oxidation of NADPH was monitored at 340 nm on a Perkin-Elmer Lamda 2S UV spectrophotometer (Perkin-Elmer). One unit of enzyme was defined as the amount of protein required to oxidize 1 μmol of NADPH/min, specific activity was defined as units per mg of protein.

**Chromatography**

**Hydrophobic Interaction**—Hi-propyl resin was equilibrated with 200 ml of 25 mM potassium phosphate buffer, pH 7.5, with 20% saturation (NH$_4$)$_2$SO$_4$ (at 4 °C) in a Pharmacia Biotech Inc. C16/70 column (15 × 1.6 cm). (NH$_4$)$_2$SO$_4$ was added to the clarified extract until 20% saturation was reached, after which the resulting extract was loaded onto the column material. A 200-ml reverse linear gradient of 20 to 0% (NH$_4$)$_2$SO$_4$ was passed over the column prior to eluting ApbA from the column with 25 mM potassium phosphate buffer, pH 7.5.

**Ion Exchange**—Q-Sepharose resin was equilibrated with 20 ml of standard buffer in a Pharmacia C10/10 column (2.5 × 1.0 cm). Fractions of the Hi-propyl eluant containing ApbA were loaded onto the column and washed with 10 ml of standard buffer. The ApbA protein was eluted from the column with 2 bed volumes of 1 M NaCl. Fractions containing ApbA as determined by SDS-PAGE were pooled, dialyzed against 100 mM potassium phosphate, pH 7.5, and concentrated in the presence of...
25% glycerol by ultrafiltration using a 10-kDa cut-off membrane, and placed at 4 °C.

**Paper Chromatography**—Descending paper chromatography was performed as described (6), using sec-butanol:propionic acid (95:5), saturated with water as the mobile phase. Chromatograms were developed on 15 × 1 cm Whatman No. 3MM paper strips for 4–5 h. The relative mobility (Rf) of KPA was determined after conversion to its semicarbazone by spraying with a solution of 0.1% semicarbazide in 0.15% sodium acetate prior to detection under ultraviolet light (330 nm). Pantoic acid was detected by spraying the dried chromatograms with a solution of 0.04% bromphenol blue in ethanol.

**Mass Spectrometry**

Fast atom bombardment mass spectra were obtained with an MS-SOTC ultra high resolution mass spectrometer (Kratos, Ltd., Manchester, United Kingdom). The spectrometer was equipped with a xenon fast atom bombardment gun (Ion Tech, Ltd., Teddington, United Kingdom). The acceleration potential was 6 kV, the source temperature was 25 °C, and no matrix was used. Data were collected and processed using a Kratos DS-55 data system.

**RESULTS AND DISCUSSION**

**Overexpression of KPA Reductase**—Early on we noted that the N-terminal His-Tag fusion of ApbA was unable to complement an apbA mutation in vivo, indicating this fusion protein was inactive. This result was consistent with a role for the N-terminal NAD+/FAD binding site in activity of the protein. Active ApbA was obtained after overexpression of the wild-type apbA allele in plasmid pT7-apbA. Expression of ApbA, as monitored by SDS-PAGE and enzyme assays, was optimal after 3 h with no further increase in specific activity up to 24 h postinduction (data not shown). The ratio of specific activities (crude extract/homogeneous protein) was 10%, suggesting that ApbA comprised approximately 10% of total cellular protein under overexpressing conditions.

**Purification of KPA Reductase**—KPA reductase was purified to homogeneity from clarified extracts of *E. coli* BL21(DE3) carrying pT7-apbA. Table I shows data obtained from a representative purification of this enzyme. An average purification began with 4 liters of cells, and 89% of the KPA reductase activity remained in the soluble fraction. The majority of proteins eluted off the Hi-propyl column in either the flow through or during the 200-ml gradient of 20 to 0% ammonium sulfate saturation. ApbA eluted in a broad peak after completion of the gradient, suggesting extensive interaction of the protein with this column matrix (data not shown). Addition of up to 50%...
ethylene glycol or up to 1.0% Triton X-100 to the buffer failed to reduce the volume needed to elute ApbA. Although we were concerned with such a strong interaction between the matrix and the protein, percent yield and specific activity measurements suggested that the activity of the protein was unaffected by the prolonged interaction with the matrix. In addition, the period of time that the protein was in dilute solution (<1 mg ml\(^{-1}\)) off this column did not affect the activity. Active fractions contained protein that was >98% homogeneous as determined by densitometric scans of 12% SDS-PAGE gels. Subsequent column chromatography on a Q-Sepharose anion exchange column concentrated ApbA and resulted in the completed purification of 45.8 mg of ApbA, a yield of approximately 78% (Table I). Although this ion exchange chromatography step resulted in little if any purification, this efficiently concentrated ApbA.

Fig. 2A shows an SDS-PAGE gel of the purification described in Table I. The presence of the ApbA protein in the active fractions was confirmed by Western analysis using anti-ApbA as shown in Fig. 2B.

N-terminal amino acid sequencing determined the first 15 amino acids of the homogeneous protein to be MKITVGLGC-GALGQLW, a perfect match to the predicted amino acid sequence of ApbA (3). This result confirmed that the purified protein was ApbA, confirmed that it was responsible for the KPA reductase activity measured, and indicated that no post-translational processing of the protein had occurred.

**ApbA Has KPA Reductase Activity**—Using the ketopantoic acid reductase assay described under "Experimental Procedures," homogeneous ApbA was shown to have a specific activity of 64.3. To determine the in vitro product of ApbA, 0.5 \(\mu\)mol of ketopantoic acid was reduced to completion by 5 units of homogeneous ApbA in the presence of 400 \(\mu\)M NADPH. The reaction was run at 42 °C for 1 h. The reaction product was isolated from substrates by paper chromatography and analyzed by fast atom bombardment mass spectrometry. This analysis identified signals with \(m/z\) ratios of 149 (\(M + H^+\)), 171 (\(M + Na^+\)), and 187 (\(M + K^+\)) and was identical to authentic pantoate.

**Characterization of the Ketopantoic Acid Reductase Activity of ApbA**—A temperature of 42 °C was determined to be the optimal in vitro temperature for ApbA at a pH of 6.0 (Fig. 3A). The pH optimum was 6.25 at a reaction temperature of 42 °C (Fig. 3B). Using 0.025 nmol (0.783 \(\mu\)g) of homogeneous enzyme under optimal reaction conditions (42 °C, pH 6.25), the specific activity of ApbA was 61 and 16 using NADPH and NADH, respectively, as electron donors (Fig. 3B). This result indicated that NADPH is likely to be the preferred source of electrons for ApbA in vivo.

HPLC gel filtration chromatography using a BioSep-SEC-S2000 column resolved a single, sharp protein peak associated with the homogeneous active ApbA fraction. This peak eluted with a volume indicating a molecular mass of 31 kDa, consistent with ApbA functioning as a monomer (data not shown).

While many characterized reductases are multimeric, a new family of monomeric enzymes including carboxyl reductases, has recently been identified and comprise the monomeric oxidoreductase family (12). Although members of this family share significant sequence similarity, ApbA showed no amino acid sequence similarity to members of this family, suggesting that ApbA may be a member of a yet uncharacterized family of monomeric oxidoreductases.

KPA reductase activities specifically, have been identified in *S. cerevisiae*, *E. coli*, *S. typhimurium*, and *P. maltophilia* 845. Because the respective enzymes in *S. cerevisiae* and *E. coli* have been only partially purified, no oligomeric data were available for these proteins although, like ApbA, each of these KPA reductases displayed a preference for NADPH (5, 13). However, the pH optimum of the *S. typhimurium* enzyme in crude extracts was reported to be 5, in contrast to the optimal pH of 6.25 determined herein for homogenous ApbA (4).

**Substrate Specificity**—A number of metabolically relevant compounds bearing structural resemblance to KPA were tested for reduction by ApbA, namely, pantoate, ketoisovalerate, oxaloacetate, pyruvate, 3-hydroxypruvic acid, \(\alpha\)-ketoglutarate, \(\alpha\)-ketobutyrate, and acetaldehyde. None of these compounds served as substrates for ApbA when provided in the assay mixture at the concentration used for ketopantoic acid (0.5 mM), reflecting the structural specificity of ApbA for its substrate.

**Electron Acceptors**—In the presence of NADPH, ApbA was unable to catalyze the reduction of a number of artificial dye electron acceptors, including dichlorophenol-indophenol, benzyl viologen, 2,3,5-triphenyltetrazolium chloride, cyanocobalamin, cytochrome c, and phenazine methosulfate.
**Kinetic Properties**—Initial velocity measurements were made using 0.129 nmol of enzyme with four concentrations of NADPH ranging from 125 to 3125 $\mu$M and four concentrations of KPA ranging from 125 to 3125 $\mu$M. Kinetic constants were determined at 42 °C, pH 6.25. The double reciprocal plots are shown in Fig. 4. The kinetic constants were determined from secondary plots of the intercepts versus the reciprocal of the fixed substrate concentration. Data were analyzed, and best fit lines were obtained using the Enzyme Kinetics Template of the Prism program (GraphPad, San Diego, CA). Kinetic constants for KPA reductase were determined using linear and non-linear regression analyses. These analyses determined that the $K_m$ constants of ApbA for NADPH and KPA were 0.776 ± 0.09 mM and 0.742 ± 0.01 mM, respectively. The $V_{\text{max}}$ value obtained from either secondary plot was 89.3 ± 2.3 units/mg. These plots are consistent with, but not conclusive of, the ApbA enzyme employing a ping-pong bi-bi reaction mechanism.

**Concluding Remarks**—Identification of KPA reductase activity for the *apbA* gene product has profound implications on our understanding of thiamine synthesis. Because *apbA* mutations cause a conditional nutritional requirement that can be satisfied by pantothenate or thiamine, knowledge of the biochemical role of this gene product will allow us to frame this activity within the context of thiamine synthesis and to further probe into the integration of the pantothenate and thiamine biosynthetic pathways.

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