The precursor to rat liver mitochondrial aspartate aminotransferase has been expressed in Escherichia coli JM105 using the pKK233-2 expression vector. This mammalian natural precursor has been isolated as a soluble dimeric protein. The amino-terminal sequence and the amino acid composition of the isolated protein correspond to those predicted from the inserted cDNA (Mattingly, J. R., Jr., Rodriguez-Berrocal, F. J., Gordon, J., Iriarte, A., and Martinez-Carrion, M. (1987) Biochem. Biophys. Res. Commun. 149, 859–865). The isolated precursor contains bound pyridoxal phosphate and shows catalytic activity with a specific activity equal to that of the mature form of the enzyme. This precursor can also be processed by mitochondria into a form with the same activities as the mature enzyme with a cleavage of a terminal extrapeptide (1). Translocation of precursor proteins across biological membranes is well established to be mediated through an initial interaction of the precursor signal sequence extrapeptide in a still unknown manner (2, 3). Furthermore, there are some indirect studies implying that some unfolding of the precursor protein may be required for membrane crossing (4, 5), and there is concern as to whether most enzyme precursors possess enzymatic activity (6, 7).

At present, naturally occurring precursor for any mitochondrial enzyme has been characterized, and the structural distinctions between precursor and mature proteins have not been studied because precursors have not been isolated in a native (nondenatured) state. Most studies have been carried out using complete in vitro expression systems of precursor proteins produced with reticulocyte lysates and monitoring radioactively labeled electrophoretic protein bands. Also, most systems were studied for the definition of general mitochondrial import characteristics of precursors of monomorphic proteins. Fundamental questions regarding properties of the structure of precursor-like molecules have recently received impetus with the preparation of an artificial system in which the presequence of yeast mitochondrial cytochrome oxidase subunit IV is fused to cytoplasmic (monomorphic) mouse dihydrofolate reductase (8). This hybrid created a model with which to begin addressing some of the problems of structure of precursor-like molecules and some of the conditions for their import into mitochondria (9–11).

With mitochondrial pyridoxal phosphate-dependent enzymes, present postulates favor either precursor synthesis with (12) or without (13) PLP coenzyme attached to it. Thus, the in vitro synthesized precursor to 4-aminobutyrate aminotransferase can apparently bind cofactors and does possess some catalytic activity (12), but chicken mitochondrial aspartate aminotransferase can be imported in vivo into mitochondria in the apo form (13). These results, however, do not address the question about how a precursor and a mature protein differ. Answering this specific question requires purification of sufficient precursor for physical studies, and one approach is to use recombinant DNA technology to produce the precursor. Even though there are recent reports of the expression of cDNA encoding for pmAspAT in Escherichia coli (14–16), the only reported attempt to purify pmAspAT from E. coli extracts was unsuccessful because the protein could not be solubilized (14). Here we report on overcoming these limitations for the purification of the precursor to rat liver mAspAT and show that the precursor is catalytically competent.

**EXPERIMENTAL PROCEDURES**

E. coli Growth and Precursor Purification—E. coli strain JM105 cells were transformed with the plasmid containing the rat pmAspAT cDNA cloned in the expression vector pKK233-2, designated pKKpmAAT as described previously (16). Five liters of M9CA medium containing ampicillin (50 μg/ml) and streptomycin (15 μg/ml) were inoculated with an overnight culture of E. coli JM105 transformed with pKKpmAAT and incubated at 37 °C with shaking. When the OD_{600} reached 0.6, chloramphenicol (65 μg/ml) was added and incubated overnight. Cells were then collected by centrifugation (5000 \times g for 15 min) washed with M9CA medium and suspended at 4-fold concentration in M9CA medium containing 0.25% succinate, 0.1% α-ketoglutarate, and 0.01% pyridoxine. After incubation for 1 h, cells were induced with 1 mm isopropyl-1-thio-β-D-galactoside for 30 min, immediately chilled in ice, and harvested by centrifugation (5000 \times g at 4 °C for 10 min). The pellet (10 g wet weight) was suspended in 40 ml of 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 10 mM succinate, 1 mM α-ketoglutarate, 16 μM PLP, 0.5 mM EDTA, 0.5 mM EGTA, 2.5 mM o-phenanthroline, 1 mM diithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Cells were disrupted by two passages through a French press at 12,000 p.s.i., and the lysate was clarified by centrifugation (140,000 \times g for 30 min). Unless
otherwise noted, this and all subsequent steps were performed at 4 °C. Nucleic acids were removed from the supernatant by dropwise addition of a solution of 10 mg/ml protease K to reach a final concentration of 1 mg/ml. After centrifugation at 12,000 × g for 20 min, the supernatant was brought to 40% saturation with solid (NH₄)₂SO₄, stirred for 30 min, and centrifuged at 12,000 × g for 20 min. The pellet was resuspended in 10 ml of 0.2 M Tris-HCl, pH 8.3, 8.0, 10 mM succinate, and 1 mM α-ketoglutarate (buffer A) containing 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM diethiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride and dialyzed overnight against the same buffer. The solution was clarified by centrifugation at 140,000 × g for 30 min and loaded on a 1.5 × 30 cm DEAE-cellulose column (Whatman DE52) equilibrated and eluted with buffer A. Fractions containing pmAspAT were pooled and loaded on an CM-Sepharose column (Pharmacia LKB Biotechnology Inc. 1 × 3 cm) equilibrated in 50 mM Tris-HCl, pH 8.3, and 100 mM KCl. In Vitro Synthesis of Radiolabeled pmAspAT—An overnight culture of E. coli JM105/pKKpmAAT, grown, concentrated, and washed as above, was washed twice with an equal volume of medium supplemented with all the L-amino acids (except cysteine and methionine) at 20 μg/ml each, 50 μg/ml ampicillin, and 25 μg/ml streptomycin. 80 ml of the 40-fold concentrated and washed cells suspended in this same medium were used for radiolabeling. Expression was induced by adding isopropyl-β-D-galactopyranoside to 1 mM and ~-[³S]methionine (ICN Trans3′-label) to 30 μCi/ml, incubating 60 min as above, adding another 30 μCi/ml L-[³S]methionine, incubating 20 min, and then harvesting the cells as described above. Radiolabeled pmAspAT was purified as described above. In Vitro Transcription and Translation—mRNA was prepared from a SacI-linearized plasmid (BSKS AAT-2) (16) containing the cDNA cloned downstream from a promoter for T3 RNA polymerase as described by the manufacturer (Stratagene Cloning Systems, La Jolla, CA) (17). Newly transcribed RNA was used to program a reticulocyte lysate protein synthesizing system containing L-[³S]methionine (18). Briefly, 1 μg of RNA was added to 35 μl of reticulocyte lysate (Promega Biotech) containing 30 μCi of L-[³S]methionine, and the mixture was incubated for 30 min at 30 °C. Protein synthesis was stopped by chilling or by the addition of 0.2 mM cycloheximide. The translation mixtures were analyzed by SDS-PAGE and fluorography. In Vitro Import of pmAspAT into Mitochondria—pmAspAT translated in vitro (40 μl) of translation mixture containing 2 × 10⁷ cpm of L-[³S]methionine incorporated into pmAspAT (10) or purified [³S]pmAspAT (6.4 × 10⁷ cpm, supplemented with 40 μl of nucleate-treated reticulocyte lysate) were mixed with an equal volume of freshly isolated rat liver mitochondria in 5 mM Tris-HCl, 0.07 M sucrose, 0.2 mM mannitol, pH 7.4 (19), containing 400 μg/ml of protein. The reactions were incubated for 30 min at 30 °C. Mitochondria were recovered by centrifugation at 10,000 × g for 10 min, washed twice with the above buffer, and dissolved in 80 μl of 2% SDS by heating at 95 °C for 5 min, and aliquots were analyzed by SDS-PAGE and fluorography.

General—Rat mAspAT was isolated from liver as described previously (20). Aspartate aminotransferase activity was measured by a coupled assay with malate dehydrogenase (21). Protein content was determined by the method of Lowry et al. (22). Absorption spectra were performed in 50 mM Tris·HCl, pH 8.3, and 100 mM KCl using a Hewlett-Packard 8452A diode array spectrophotometer. SDS-PAGE was performed in 10% polyacrylamide slab gels according to Laemmli (23). The gels were prepared for fluorography using an enhanced silver staining kit from Research Products International following the recommendation of the manufacturer. Electrophoretic transfer on nitrocellulose membrane was performed according to Towbin et al. (24), and proteins were immunodetected with the biotinstreptavidin-alkaline phosphatase system (Bethesda Research Laboratories, using antiserum against mouse AspAT or phosphorpyridoxylpolylysine complexes raised in rabbit).

Amino acid analysis was carried out after hydrolysis of the samples in 6 N HCl at 110 °C for 24 h. The T-terminal sequence analysis was performed with the assistance of Ben Madden at Mayo Clinic, Rochester, MN, using an Applied Biosystems, Inc. gas phase sequencer.

RESULTS

Purification and Chemical Characterization of pmAspAT Expressed in E. coli—Successful purification of an expressed protein depends upon the ability to solubilize it in a native state. In order to determine the solubility of our precursor, we used Western blots to follow pmAspAT in the developmental stages of the purification protocol. In earlier attempts at expression of the highly homologous pmAspAT from chicken heart, the precursor produced was soluble only in strongly denaturing buffers (14). However, in our extraction buffer, most of the pmAspAT is soluble and is recovered in the supernatant of the high speed centrifugation. Western blots after SDS-PAGE using anti-pig heart AspAT detect the presence of pmAspAT in crude extracts of E. coli harboring the expression plasmid (16). Yet, these antibodies also cross-react with some other protein fragments but not with E. coli aspartate aminotransferase, which is present in our E. coli strains but has a different electrophoretic mobility. The bulk of E. coli proteins are removed in the first stages of the preparation (Fig. 1 and Table 1). Among the most functionally important proteins removed is E. coli aspartate aminotransferase; this enzyme has been reported to be soluble in 40% ammonium sulfate and to bind to DE52-cellulose at pH 8.3 (25). We also observed that these two procedures removed all aspartate aminotransferase activity from control extracts (JM105/pKK233-2 without the rat pmAspAT cDNA).

The subsequent chromatographic fractionations are very efficient, ultimately yielding an almost 10,000-fold purification. 46% of the transaminase activity in the 40% ammonium sulfate precipitate was recovered as the pure protein. This amounts to 0.01% of the total E. coli protein in the crude lysate. The purified protein shows a single band on both Coomassie and silver-stained SDS-PAGE. Western blotting

FIG. 1. SDS-PAGE analysis of the protein purification stages. Fractions were analyzed by 10% SDS-PAGE at selected steps in the purification of pmAspAT from isopropyl-β-D-galacto-pyranoside-induced pKKpmAAT/JM105. Lane 1, French press supernate; lane 2, ammonium sulfate pellet (0–40%) after dialysis and centrifugation; lane 3, DEAE-cellulose fractions; lane 4, CM-cellulose fractions; lane 5, Superose 12 fast protein liquid chromatography fractions; lane 6, mAspAT purified from rat liver. The gel was stained with Coomassie Blue.

| Volume (ml) | Protein (mg) | Activity (units) | Specific activity (units/mg) |
|------------|-------------|-----------------|----------------------------|
| (NH₄)₂SO₄ precipitate | 10 | 139.00 | 26.3* | 0.2 |
| DEAE-cellulose | 15 | 6.00 | 22.5 | 3.8 |
| CM-cellulose | 8 | 1.50 | 16.5 | 11.0 |
| Superose 12 | 1 | 0.05 | 11.0 | 220.0 |

* SDS-PAGE reveals a minor contamination with intrinsic E. coli aspartate aminotransferase.
and immunodetection with pig mAspAT antiserum also shows a single band.

The amino acid composition of the isolated protein determined on preparations greater than 95% pure corresponds (Table II) to that predicted by the pmAspAT sequence and differs in the appropriate residues from the composition of rat liver mAspAT obtained under identical experimental conditions.

The precursor protein isolated from E. coli was also subjected to N-terminal sequence analysis to determine whether any amino acid residues were removed subsequent to translation. 30 cycles of automated Edman degradation were performed, and the sequence Ala-Leu-Leu-His-Ser-Gly-Arg-Val-Leu-Ser-Gly-Met-Ala-Ala-Ala-Phe-His-Pro-Gly-Leu-Ala-Ala-Ala-Ala-Ala-Ser-Arg-Ala-Ser-Ser was obtained. This sequence is that predicted by the cDNA sequence of pmAspAT (16) and indicates that the complete cDNA coding region for the precursor signal peptide region has been translated and differs in the appropriate residues from the composition of rat liver mAspAT obtained under identical experimental conditions.

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Catalytic Activity and Coenzyme Content of Isolated pm-AspAT—The purified precursor contains transaminase activity with a specific catalytic activity of 220 units/mg which is identical to that for mature mAspAT isolated from rat liver (20). The \( K_m \) values for the substrates L-aspartate, 0.35 mM, and \( \alpha \)-ketoglutarate, 1.1 mM, are also indistinguishable from those for the mature enzyme as measured by us under identical conditions (0.33 and 1.15 mM, respectively). The pm-AspAT concentration was determined from the absorbance at 280 nm, assuming that the molar absorption coefficient of the precursor is the same as that of the mature rat mAspAT. Validating this assumption is the absorption ratio 280/358 nm which is the same for both proteins. It is unlikely that any contaminating species not detected by SDS-PAGE or fast protein liquid chromatography could have a transaminase catalytic activity high enough to account for the observed specific activity.

The catalytic activity of the precursor implies two properties that are easily demonstrated: 1) a dimeric structure, because each active site is symmetrically composed of elements from both subunits (26) and 2) presence of PLP at the active site. In the last purification step, the precursor elutes from the Superose 12 column with the same retention volume as the mature, dimeric enzyme from rat liver. Additionally, the precursor exhibited dimeric behavior even in the crude extract. This was shown by fractionation on sucrose gradients and analysis by Western blotting (data not shown). Similar elution profiles have been reported in gel filtration experiments of the rat pmAspAT produced in reticulocyte lysate (27).

We used two approaches to demonstrate PLP binding. If treated with NaBH\(_4\) prior to SDS-PAGE and Western blotting, the precursor band reacts with antiserum raised to the phosphopyridoxyl moiety (Fig. 2). (NaBH\(_4\) reduces the internal Schiff's base which would otherwise be hydrolyzed, releasing the cofactor upon denaturation of the holoenzyme.)

![Fig. 2. Western blot analysis of purified pmAspAT.](image)

![Fig. 3. UV absorption spectrum of pmAspAT.](image)

![Fig. 4. In vitro import of pmAspAT into mitochondria. A, in vitro translated precursor; B, purified radiolabeled precursor. Lanes 1, translation mixture or purified precursor not incubated with mitochondria (15% of samples present in each incubation); lanes 2, mitochondrial pellets. \( p \) indicates the position of pmAspAT, and \( m \) indicates that of mAspAT.](image)

### Table II

| Amino Acid | Observed\(^a\) | Predicted\(^a\) | Amino Acid | Observed\(^a\) | Predicted\(^a\) |
|-----------|---------------|----------------|-----------|---------------|----------------|
| Ala       | 41            | 43             | Lys       | 31            | 30             |
| Arg       | 23            | 22             | Met       | 9             | 12             |
| Asx       | 38            | 37             | Phe       | 21            | 21             |
| Cys       | ND\(^b\)      | 7              | Pro       | ND\(^b\)      | 20             |
| Glx       | 39            | 39             | Ser       | 29            | 29             |
| Gly       | 39            | 39             | Thr       | 19            | 19             |
| His       | 11            | 11             | Trp       | ND\(^b\)      | 7              |
| Ile       | 18            | 19             | Tyr       | 11            | 12             |
| Leu       | 36            | 36             | Val       | 28            | 29             |

\(^a\) The results are based on a subunit \( M_r = 47,000 \) for pmAspAT.

\(^b\) ND, not determined.
more direct proof of PLP binding, however, is the appearance of an absorption band at 358 nm in the UV-visible spectrum of the precursor (Fig. 3). This is characteristic of PLP bound in its active form as a Schiff's base. In one-half of a transaminase reaction, the bound PLP cofactor reacts with an amino acid substrate to produce the pyridoxamine form of the cofactor which absorbs at a different characteristic wavelength. In our pmAspAT preparation, the transaminase half-reaction is readily seen in the immediate conversion to a 335-nm band by addition of L-aspartate (Fig. 3) or cysteine sulfinate.

In Vitro Import of pmAspAT into Isolated Mitochondria—Previous studies have shown that when a rabbit reticulocyte lysate-programmed translation mixture containing radiolabeled pmAspAT is incubated with isolated mitochondria, the precursor is taken up and converted to the mature form (15, 28, 29). In vitro transcription-translation of the pmAspAT cDNA yields a single band in SDS-PAGE with an apparent Mr = 47,000, the expected size for pmAspAT (16), and co-migrates with the precursor purified from E. coli extracts synthesized in vivo (Fig. 4, lanes A1 and B1). Furthermore, both the in vitro translation product and the purified precursor are imported into mitochondria and processed to mature-sized species when incubated with freshly isolated rat liver mitochondria (Fig. 4, lanes A2 and B2).

DISCUSSION

The aim of the studies reported here was the isolation of a precursor protein for a mitochondrial enzyme, pmAspAT. In the current findings, perhaps our most surprising observation is that this precursor is largely soluble. This is in marked contrast to the previously reported insolubility of a very similar protein (14). High levels of expression of foreign proteins in E. coli often result in the formation of large amounts of insoluble protein relative to the soluble product (30). Presumably, partially folded proteins aggregate at these high concentrations. The relatively low levels of expression that both we and Jaussi et al. (14) observe might be expected to minimize such precipitation by giving the nascent polypeptide opportunity to fold properly. The amino acid sequence homology of the mature part of the chicken pmAspAT (31) is 86% relative to that of the rat precursor. However, the aminoterminal extension of the chicken pmAspAT (32) is seven residues shorter than that of the rat pmAspAT, so it is possible that this difference alone could explain the dissimilar solubility of the two precursors. This would suggest a strong influence of the presequence on the precursor protein structure, or the difference may simply lie in the very different types of expression systems employed. Furthermore, Jaussi et al. (14) used a temperature-induced derepression of the λ P1 promoter to express the chicken pmAspAT. Our thermal stability data indicate (data not shown) that at 42°C the purified rat pmAspAT is labile. This instability may be exacerbated in vivo in the early stages of protein folding, leading to the formation of insoluble species. In our early attempts to purify rat pmAspAT from the pKKpmAAT expression system using a low ionic strength, pH 6.8 buffer, we obtained a preponderance of insoluble pmAspAT and a remarkable increase in solubility was obtained when the ionic strength of the lysis buffer was raised.

A previous study of the precursor to another PLP-dependent enzyme, 4-aminobutyrate aminotransferase, concluded that the specific activity could approach that of the nature enzyme (12). This was based on an estimate of the precursor's concentration in a reticulocyte lysate translation mixture. The concentration was calculated from the initial specific radioactivity of the [35S]methionine, the relative amount of radiolabel incorporated into immunoprecipitable precursor, the assumed number of methionine residues in the precursor, and the radioactivity determined by autoradiography. Such calculations produced a rough estimate because, in addition, the recovery of precursor in the immunoprecipitate and the sequence of the precursor were unknown. However, those and similar results for other enzyme precursors (7, 33) showed that precursor catalytic activity is possible. Using a direct approach, we can now say that this particular precursor has, within an experimental uncertainty of ±10%, the same specific activity and substrate Kₜ values as the mature enzyme.

At first glance, an active precursor implies properties associated with proteins that cannot be imported. The foremost of these properties is a rigid three-dimensional structure. In several precursors, ligand binding, which tightens the protein structure, has been shown to inhibit import (8, 34), and the binding of PLP, required for activity, is known to impart considerable structural organization to the mature protein (26). Thus, if for mitochondrial import protein unfolding is required, PLP would be expected to prevent or retard precursor import (4, 5). However, we observe mitochondrial import and processing of the purified precursor in the holo form (Fig. 4), although at this time we do not know whether this precursor protein has the same physical properties as precursor produced in vivo in the cytoplasm. An in vivo study of chick embryo fibroblasts indicates an accumulation of mAspAT apoenzyme in the mitochondria of cells deprived of PLP (13). Those results imply that in these cells newly synthesized precursor probably exists mostly in an apoenzyme form and can be imported as such but do not exclude a possible holoprecursor import mechanism. Perhaps the rest of the required import components (reticulocyte lysate and mitochondrial membrane) create an environment in which the transit (signal) peptide can facilitate precursor unfolding, regardless of its coenzyme content. In any event, purified holoprecursor is a "substrate" for mitochondrial import and processing.

In conclusion, this is the first report of the isolation of an intact, catalytically competent precursor protein containing bound coenzyme. The ability to purify, characterize, and, potentially, modify a naturally occurring precursor produced by recombinant DNA techniques provides a new model for investigating the intricacies of mitochondrial import. Furthermore, the availability of purified pmAspAT allows one to design experiments directed toward understanding the effect of the presequence on the properties of the mature domain.

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