Purification and General Properties of the DNA-binding Protein (P16) from Rat Liver Mitochondria

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ABSTRACT The mitochondrial DNA-binding protein P16 was isolated from rat liver mitochondrial lysates by affinity chromatography on single strand DNA agarose and separated from DNA in the preparation by alkaline CsCl isopycnic gradients. The top fraction of the gradients contained a single polypeptide species ($M_w \approx 15,200$) based upon SDS PAGE. Digestion of single strand DNA-bound P16 with proteinase K produced a protease-insensitive, DNA-binding fragment ($M_w \approx 6,000$) that has been purified by essentially the same procedures used for intact P16. The partial amino acid compositions for P16 and the DNA-binding fragment were obtained by conventional methods. Analysis of subcellular fractions revealed that nearly all of the cellular P16 was located in the mitochondria and that only trace amounts of protein of comparable electrophoretic mobility could be isolated from the nuclear or cytoplasmic fractions. The labeling of P16 with $[^{35}S]$methionine in primary rat hepatocyte cultures was inhibited by more than 90% by the cytoplasmic translation inhibitor cycloheximide, but unaffected by the mitochondrial-specific agent chloramphenicol. These results indicate that P16 is synthesized on cytoplasmic ribosomes and imported into the mitochondria. The addition of purified P16 to deproteinized mitochondrial DNA resulted in the complete protection of the labeled nascent strands of displacement loops against branch migrational loss during cleavage of parental DNA with $SstI$, thus providing strong evidence that P16 is the single entity required for this in vitro function. Incubation of P16 with single strand $\Phi X174$ DNA, double strand (RF) $\Phi X174$ DNA, or Escherichia coli ribosomal RNA and subsequent analysis of the nucleic acid species for bound protein indicated a strong preference of P16 for single strand DNA and no detectable affinity for RNA or double strand DNA. Examination of P16-single strand $\Phi X174$ DNA complexes by direct electron microscopy revealed thickened, irregular fibers characteristic of protein-associated single strand DNA.

A previous study from our laboratory (1) identified P16 ($M_w \approx 16,000$) as the single detectable polypeptide present in native mtDNA-protein complexes isolated from rat liver mitochondria. One function of the bound protein component was to stabilize the displacement loop (D-loop) structure by protecting the nascent strand of the D-loop from branch migration upon parental strand cleavage in vitro. In the accompanying paper (2), P16 was found to bind exclusively to the displaced single strand of normal and expanded D-loops and to the single strand gap segment of molecules with the characteristics of $\beta$-gapped circles. Thus, in addition to stabilization of the D-loop structure, P16 probably functions at all stages of the asymmetrical replication cycle of mtDNA. Further evidence from that study indicated that there were an average of $\sim 49$ P16 molecules per mtDNA in the bound population composed predominantly of D-loop DNA.

The work presented here describes the isolation of P16 and a protease resistant, DNA-binding domain of P16 from rat liver mitochondria and compares these with regard to amino acid composition. Evidence is provided that, like most other mitochondrial proteins, P16 is synthesized on extramitochondrial cytoplasmic ribosomes. In addition, the ability of purified P16 to bind single strand DNA and to restore stability to the triplex D-loop structure is examined.

MATERIALS AND METHODS

Materials: Male Sprague-Dawley rats weighing 150–250 g were purchased from Flow Laboratories, Inc. (Dublin, VA). [Methyl-$^3$H]dThd and [35S]methionine were purchased from ICN Chemical and Radioisotope Division.
Abbreviations used in this paper: D-loop, displacement loop; mtDNA, mitochondrial DNA; ssDNA, single strand DNA; TE, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4; TKM, 50 mM Tris-HCl, 1 mM EDTA, pH 7.4; TKM buffer, 50 mM Tris-HCl, 1 mM EDTA, pH 7.4; TKM. The suspension was then shaken for 15 min in a 70°C water bath, cooled on ice, and the precipitate was removed by centrifugation at 12,000 g for 15 min. The clarified supernatant was mixed with gentle rotation at 4°C with 5 ml of sucrose-DNA agarose (ssDNA agarose) that had been equilibrated in 0.5 M NaCl, TE.

Isolation of P16: Unless otherwise noted, all procedures were carried out at 0-4°C. Mitochondria were isolated from 50-60 g of rat liver by differential centrifugation as described previously (3). The final mitochondrial pellet was resuspended with homogenization in ~30 ml of 0.5 M NaCl, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4 (0.5 M NaCl, TE). The resuspended mitochondrial fraction was broken by sonication with six 20-s bursts at level six of a Heat Systems-Ultrasonics Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc., Farmingdale, NY) equipped with a standard microtip. The mitochondrial suspension was held on ice during the bursts and allowed to cool for 30 s between bursts. Lysis of the sonicated mitochondria was made more complete by the addition of β-octylglucopyranoside to a final concentration of 33 mM.

The suspension was then shaken for 15 min in a 70°C water bath, cooled on ice, and the precipitate was removed by centrifugation at 12,000 g for 15 min. The clarified supernatant was mixed with gentle rotation at 4°C with 5 ml of sucrose-DNA agarose (ssDNA agarose) that had been equilibrated in 0.5 M NaCl, TE. Before use, the ssDNA agarose was preswollen with 20 ml aliquots of 7% (wt/vol) NH4OH, pH 12.2 followed by equilibration with 80 ml of 30-mi aliquots of 0.5 M NaCl, TE. After overnight mixing, any unbound material was washed from the ssDNA agarose by eight washes with 0.5 M NaCl, TE. The ssDNA agarose was then packed into a 1.2-cm diam column with washes of 50 ml of 0.5 M NaCl, TE. The eluent of each subfraction was then analyzed for the presence of P16 by SDS-PAGE.

To determine the binding preference of P16, ~5 to 7 μg of ssDNA agarose equilibrated in 0.5 M NaCl, TE. Unbound material was removed from the ssDNA agarose by 10 washes with 0.5 M NaCl, TE followed by five washes with 1 M NaCl, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4 and five washes of 2.0 M NaCl, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4. Fractions corresponding to the peak of absorbance exhibited by the elution of the mitochondrial subfraction were collected for the nuclear and cytoplasmic subfractions. The eluent of each subfraction was then analyzed by SDS-PAGE for the presence of P16.

Properties of P16: P16 was isolated from the supernatant of rat liver homogenates by differential centrifugation and equilibrated in 0.5 M NaCl, TE. Unbound material was removed from the ssDNA agarose by 10 washes with 0.5 M NaCl, TE followed by five washes with 1 M NaCl, 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4. Fractions corresponding to the peak of absorbance exhibited by the elution of the mitochondrial subfraction were collected for the nuclear and cytoplasmic subfractions. The eluent of each subfraction was then analyzed by SDS-PAGE for the presence of P16.
Complexes to be analyzed by direct electron microscopy were prepared between single strand φX174 and P16, which had been dialyzed against 0.20 M NaCl. 0.02 M sodium phosphate, pH 7.4. Samples (50-100 μl) containing P16 and single strand φX174 in a 6.7:1 protein/DNA mass ratio were incubated at room temperature for 2 h. To assay for spurious binding or aggregation of added protein onto the DNA using these procedures, an incubation of single strand φX174 and ribonuclease A was carried out under identical conditions. Samples were prepared for direct electron microscopy as previously described (12, 13). This included fixation by the two-step formaldehyde/glutaraldehyde procedure, direct mounting onto glow charged carbon films, washing, drying, and shadowcasting with tungsten. Complexes were examined and photographed on a Hitachi HU 12 electron microscope.

Complexes between P16 and SDS/phenol deproteinized H-Q mtDNA (3) were formed in 50-100 μl of a buffer containing a slightly lowered NaCl concentration (0.1 M NaCl, 0.02 M sodium phosphate, pH 7.4) to facilitate subsequent restriction endonuclease cleavage. Each sample was incubated for 1 h at room temperature prior to cleavage. Samples were then adjusted to 6 mM MgCl2 and 6 mM β-mercaptoethanol followed by the addition of at least a fivefold excess of restriction endonuclease SstI. Samples were digested 1 h at 37°C, cooled on ice, and layered directly onto density gradients of 5-20% (wt/vol) sucrose containing 0.5 M NaCl, TE. The gradients were centrifuged at 4°C for 165 min at 49,000 rpm in a Beckman SW 50.1 rotor and fractionated dropwise from the bottom. Radioactivity was determined by scintillation counting of each 200-μl fraction directly in Hydrofluor.

**SDS Polyacrylamide Gradient Gel Electrophoresis:** Protein samples were dialyzed against 0.2 M ammonium bicarbonate and concentrated to dryness in a Savant Speed Vac Concentrator. The dried samples were dissolved in 100 μl of 5% (wt/vol) glycerol, 3% (wt/vol) SDS, and 0.0625 M Tris-HCl, pH 6.8 (14), and heated for 5 min in a boiling water bath. Electrophoresis was carried out on exponential gradient gels of 3-20% polyacrylamide as previously described (1). The protein molecular weight standards were phosphorylase B, 92,500; BSA, 66,000; ovalbumin, 43,000; α-chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; lysozyme, 14,300; cytochrome c, 12,300; and bovine trypsin inhibitor, 6,200. The Coomassie Blue staining procedure was previously described (1).

**Amino Acid Analysis:** The top 1-ml fraction from an alkaline CsCl isopycnic gradient containing the sample as well as from a blank gradient were dialyzed extensively against 0.2 M ammonium bicarbonate. Samples were then evaporated to dryness in a Savant Speed Vac Concentrator (Savant Instruments, Inc.). To aid the removal of the residual ammonia present after the dialysis samples were re-evaporated and a second addition of distilled water was made. After heating for 5 min and drying as before, 0.2 ml of 6 M constant boiling HCl was added. Samples were hydrolyzed in vacuo for 24 h at 110°C. After drying, the samples were dissolved in 100 μl of 0.2 M sodium citrate, pH 2.2, and applied to a Durrum amino acid analyzer (Durrum Instruments, Inc., Palo Alto, CA) equipped with a Varian CDSIII integrator (Varian Associates, Inc., Palo Alto, CA). The amino acid composition was determined from Rf values on SDS polyacrylamide gradient gels, as noted by the shift in λmax toward 260 nm in fractions 2 through 5 (data not shown). The protein present in each 1-ml gradient fraction was visualized on an SDS polyacrylamide gradient gel (Fig. 1). The protein present in each 1-ml gradient fraction was visualized on an SDS polyacrylamide gradient gel (Fig. 1). P16 (M, ≈ 15,200) was the only protein detectable by Coomassie Blue staining. The majority of the P16 was present in the top 2 ml of the gradient with rapidly diminishing amounts in the lower DNA-containing fractions. To ensure that the final P16 preparation was free of contaminating DNA, all P16 used in further studies was recovered from the top 1-ml fraction only. The average yield of purified P16 was 0.38 μg/ml of rat liver.

The partial amino acid composition of purified P16 is presented in Table I. Perhaps the most noteworthy feature is the highly charged nature of this protein due to the high content of Asx, Glx, and Arg. However, because the isoelectric point of P16 was previously found to be 7.6-7.8 (1), about half of the glutamic acid and aspartic acid residues reported here were likely to have been in the amide form prior to hydrolysis. A molecular weight of 14,364 for P16 can be estimated from this amino acid composition. This correlates well with the molecular weight of 15,200 determined from 14,364 for P16 can be estimated from this amino acid composition. This correlates well with the molecular weight of 15,200 determined from Rf values on SDS polyacrylamide gradient gels, especially in light of the fact that tryptophan, cysteine, and proline were not included in the compositional analysis.

Digestion of DNA-bound P16 with proteinase K yields a protease-resistant fragment (M, ≈ 6,000) that retains the capacity to bind single strand DNA (2). Purified preparations of the fragment exhibit a single band (M, ≈ 6,000) by Coomassie Blue staining in SDS polyacrylamide gels (data not shown). The amino acid composition of this fragment is also shown in Table I. As in the case for the intact P16, the fragment exhibits a high content of the charged amino acids Asx, Glx, and Arg. The total number of amino acids of the fragment is ~40% that of intact P16. However, several amino acids are present in the fragment in disproportionately high concentrations. These include serine, glycine, tyrosine, phenylalanine, and arginine as well as the single histidine.

**RESULTS**

**Isolation of P16**

Isolated mitochondria were broken by sonication and further lysed by the detergent β-D-octylglucopyranoside. Clarification of this suspension after heating to 70°C produced a mitochondrial extract that was then mixed directly with ssDNA agarose in the presence of 0.5 M NaCl. The protein remaining bound to the ssDNA agarose after washing with 0.5 M NaCl was removed as a single peak by alkaline elution. The presence of contaminating DNA in this peak was effectively removed from the P16 preparation by centrifugation in an alkaline CsCl isopycnic density gradient. Under the alkaline conditions employed, all protein-DNA associations were abolished, allowing free P16 to be buoyed to the top of the gradient as indicated by an absorbance peak at 280 nm in fraction 1, whereas the more dense DNA approached its characteristic buoyant density near the bottom of the gradient.

**FIGURE 1** SDS polyacrylamide gel analysis of each 1-ml fraction of an alkaline CsCl isopycnic density gradient. The DNA-containing P16 preparation from ssDNA agarose was centrifuged to equilibrium in alkaline CsCl as described under Materials and Methods. The gradient was fractionated from the top into 5 1-ml fractions. Each fraction was neutralized and extensively dialyzed against 0.2 M ammonium bicarbonate, pH 7.5. Fractions 1 through 5 are in order of increasing density and represent fractions from the top to the bottom of the CsCl gradient. Samples were evaporated to dryness, resuspended by heating in an SDS-containing buffer, and electrophoresed on a 5-20% polyacrylamide gradient gel as described in Materials and Methods. Lane 1, 1/20 of the top CsCl fraction; lanes 2-5, 1/2 of CsCl fractions 2-5, respectively.
**Table I**

Amino Acid Composition of P16 and the DNA-binding, Protease-resistant Fragment (M, ~ 6,000) of P16

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>P16</th>
<th>Proteinase K-resistant fragment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid/Asparagine</td>
<td>17.5 (18)</td>
<td>4.7 (5)</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.8 (5)</td>
<td>2.1 (2)</td>
</tr>
<tr>
<td>Serine</td>
<td>5.1 (5)</td>
<td>3.1 (3)</td>
</tr>
<tr>
<td>Glutamic acid/Glutamine</td>
<td>19.6 (20)</td>
<td>6.0 (6)</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.2 (9)</td>
<td>5.1 (5)</td>
</tr>
<tr>
<td>Alanine</td>
<td>8.7 (9)</td>
<td>3.1 (3)</td>
</tr>
<tr>
<td>Valine</td>
<td>14.3 (14)</td>
<td>4.3 (4)</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.1 (3)</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.8 (7)</td>
<td>2.7 (3)</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.0 (9)</td>
<td>3.9 (4)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.4 (3)</td>
<td>1.6 (2)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.0 (4)</td>
<td>2.0 (2)</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.4 (1)</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.9 (7)</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td>Arginine</td>
<td>12.4 (12)</td>
<td>5.8 (6)</td>
</tr>
</tbody>
</table>

Duplicate samples of P16 and quadruplicate samples of the DNA-binding fragment were hydrolyzed in vacuo for 24 h at 110°C in 6 M HCl. After correcting for the blanks, the amino acid compositions were determined by comparison to a standard containing 1 nmol of each amino acid. Results are expressed as number of residues. The numbers in parentheses are the nearest integers.

* M, ~ 6,000.

Functional Reconstitution of Deproteinized mtDNA with Purified P16

mtDNA-P16 complexes, formed by incubation of SDS/phenol-deproteinized 3H-mtDNA with purified P16, were digested with the restriction endonuclease SstI which cleaves rat mtDNA at a single site (15). The resulting linearized product was then analyzed by sucrose density gradient centrifugation. The sedimentation profile (Fig. 2) demonstrates that SstI cleavage of SDS/phenol-deproteinized mtDNA in the absence of added P16 resulted in a peak of radioactivity at 7S, reflecting the branch-migrational release of the 7S 3H-nascent strand from the parental molecule. In contrast, the cleavage of the reconstituted complex with SstI resulted in a peak of radioactivity at 24S, corresponding to the s value of linearized, full-length mtDNA. This indicates that the 7S 3H-nascent strand of the D-loop remained associated with the linearized mtDNA-P16 complex. These results are identical with those of our earlier studies on native mtDNA-protein complexes (1).

Determination of the Nucleic Acid-binding Preference of P16

Samples of ribosomal RNA, single strand φX174 viral DNA, and double strand φX174 RF DNA were incubated with purified P16 and re-isolated by band sedimentation in 5-20% sucrose gradients. The nucleic acid bands were pooled and analyzed for the presence of bound P16 on an SDS polyacrylamide gradient gel electrophoresis as described in Materials and Methods. The lanes correspond to samples from gradients containing (A) P16 alone; (B) ssDNA + P16; (C) dsDNA + P16; (D) ribosomal RNA + P16. Molecular weight standards are shown on the left.

Subcellular Localization of P16

Separate nuclear, cytoplasmic, and mitochondrial fractions were prepared from equal amounts of rat liver. The P16 isolation procedure was then carried out under identical conditions on each of the subcellular fractions. The pH 12 eluents
from each of the three ssDNA agarose columns were then analyzed by SDS PAGE. A comparison of the densitometer scans of the gel lanes is given in Fig. 5. It is apparent that the vast majority of P16 which can be isolated by this procedure was present in the mitochondria (>90%) with only trace amounts of protein of comparable electrophoretic mobility in the nuclear and cytoplasmic fractions.

**Determination of the Intracellular Site of Synthesis of P16**

To determine whether P16 was synthesized on cytoplasmic or mitochondrial ribosomes, cultured rat hepatocytes were allowed to carry out protein synthesis in the presence of [³⁵S]methionine. During this incubation, cytoplasmic or mitochondrial protein synthesis was selectively inhibited using cycloheximide or chloramphenicol, respectively. P16 was then isolated from the cultured rat hepatocytes by a modification of the normal P16 isolation procedure and analyzed on an N,N'-diallyltartardiamide cross-linked SDS polyacrylamide gradient gel. As shown in Fig. 6, similar quantities of P16, as judged by Coomassie Blue staining, were isolated from control, cycloheximide-treated, and chloramphenicol-treated hepatocytes. When the P16 bands were excised from each lane and the amount of incorporated radioactivity determined, it was found that chloramphenicol caused no reduction in the amount of [³⁵S]methionine incorporated into P16. In contrast, significant inhibition of [³⁵S]methionine incorporation was seen when cycloheximide was present. These results indicate that P16 is synthesized on cytoplasmic ribosomes and transported by some as yet undetermined mechanism into the mitochondria.

**DISCUSSION**

A DNA-binding protein (P16) was isolated from crude mitochondrial lysates by ssDNA agarose chromatography and separated from contaminating DNA in alkaline CsCl isopycnic density gradients. The purified protein appeared as a single Coomassie Blue-staining band with an apparent molecular weight of 15,200 on an SDS polyacrylamide gradient gel. The most efficient method found for elution of P16 from the ssDNA agarose was the use of NH₄OH at about pH 12. Less severe conditions such as 8 M urea or NaCl concentrations up to 5 M were only partially effective. Based upon the extreme measures necessary to disrupt the P16-ssDNA association, it is evident that this protein binds very tenaciously to single strand DNA. Other DNA-binding proteins from a variety of sources are known to have very high affinity for single strand DNA, but in most cases such complexes can generally be disrupted by less harsh conditions such as 2 M NaCl (16). The durability of P16 itself is evident from the observation that the isolated protein purified by our procedures retains the capacity to rebind single strand DNA. As shown by sucrose density gradient analyses, purified P16 exhibited preferential, and perhaps exclusive, binding to single strand DNA.
The single strand DNA-binding proteins of each fraction were collected on ssDNA agarose, washed with 2.0 M NaCl, and eluted with 7% (wt/vol) NH₄OH. The eluent was subjected to 5–20% polyacrylamide gradient gel electrophoresis with a densitometric scan of the gel lanes shown.

Based upon the stability of single strand DNA-P16 complexes against high salt concentrations, it is likely that hydrophobic interactions play a major role in the binding mechanism. Comparison of the amino acid compositions of intact P16 and the protease-resistant, DNA-binding fragment (Mr 6,000) revealed the presence of a disproportionately high concentration of several amino acids in the fragment. These included, among others, the aromatic amino acids tyrosine and phenylalanine. There is considerable evidence (16) that these aromatic residues are directly involved in the binding of T4 gene 32 protein and fd gene 5 protein to single strand DNA. Our results are consistent with the possibility that a similar binding mechanism also occurs in DNA-P16 complexes.

It is likely that P16 exhibits little or no specificity for base sequence since it was shown to bind virtually all segments of displaced heavy strand DNA in native complexes (2). Moreover, complexes formed between purified P16 and single strand φX174 DNA were clearly evident by direct electron microscopy.

Reconstitution of P16 with completely deproteinized mtDNA restored the protection of the labeled nascent strand of the D-loop against branch migrational loss following restriction endonuclease-cleavage of the parental strands. This is also a characteristic of native complexes and confirms that P16 is the single component necessary for the maintenance of the integrity of the D-loop structure. In light of this unusual characteristic, it is likely that P16, in contrast to many other single strand DNA-binding proteins (16), inhibits rather than promotes reannealing of double strand DNA. The relationship of this function identified in vitro and other possible functions of P16 with respect to the novel asymmetric mechanism of mtDNA replication are discussed in the accompanying manuscript (2). The nascent strand of the D-loop is known to have an extremely high turnover rate (19), yet >70% of rat liver mtDNA molecules can be isolated in the D-loop form (2). If the overall maintenance of this unique structure is related only to replication, it is intriguing that the vast majority of the nascent strands are never extended to full length molecules. The suggestion that the D-loop may also be involved in transcriptional control derives from the fact that transcription of both the heavy and light strands initiates at specific promoters that reside very near the D-loop region (20). It is possible that the continued maintenance of the D-loop in an opened form by the association of P16 with the displaced single strand may be necessary for the recognition or conformational exposure of the transcriptional control sites in vivo.

In cultured rat hepatocytes the translation of P16 was inhibited by cycloheximide but unaffected by chloramphenicol indicating that P16 is synthesized on cytoribosomes presumably from nuclear-encoded mRNA. The very small amounts of protein obtained from the nuclear and cytoplasmic fractions having electrophoretic mobilities comparable with P16 are likely to have arisen from small amounts of mitochondria contaminating the nuclear fraction and from P16 released into the cytoplasm during cell disruption. Thus,
it is likely that P16 is indeed a mitochondrial protein and that it is imported into the organelle from an extra-mitochondrial site of biosynthesis.

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