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Characterization of a *Xenopus laevis* mitochondrial protein with a high affinity for supercoiled DNA

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ABSTRACT

A DNA binding protein of 31 Kd -mtDBPC- has been isolated from *X. laevis* oocyte mitochondria. It is present in large amounts in the organelle and does not show any enzymatic activity. Its binding to the superhelical form of a DNA is higher than for any other form, or for RNA. No sequence specificity could be found for any mtDNA fragments tested, including both origins of replication. It is able to introduce superhelical turns into relaxed circular DNA in the presence of a topoisomerase I activity. It could be a component of the mitochondrial nucleoids.

INTRODUCTION

Mitochondrial nucleoids (mtDNA-protein complexes) have been found in various organisms but the proteins bound to the mtDNA have never been clearly identified. A histone-like protein of 20 Kd, HM, has been reported in yeast mitochondria, it is able to introduce superhelical turns in relaxed circular DNA molecules (1) but it was not recovered from purified nucleoids (2) and it is not yet known if it could have a structural role in the organization of the nucleoid. In *Xenopus laevis* oocyte mitochondria we have shown that mtDNA is packaged in a compact beaded structure which is probably associated with the membrane (3). The characterization of the protein components of the beads was made difficult by the presence of membrane fragments; however one acid-soluble protein (28 Kd) was only present in the nucleoid fractions and therefore seemed to be a specific constituent of the nucleoid structure. When mtDNA molecules are in a replicative form the single-stranded part of the D-loop is covered with a protein, mtSSB, which could be isolated from both nucleoid fractions and whole mitochondria (4, 5). This mtSSB is probably similar to the Pl6 protein described in rat liver mitochondria (6).

This work was carried out to purify from *X. laevis* oocyte mitochondrial extracts DNA-binding protein(s) having properties expected for the protein component(s) of the beaded nucleoid and most importantly a specific affinity

for supercoiled DNA. We report here the isolation of a DNA-binding protein, present in a rather large amount in the mitochondria, which binds preferentially to supercoiled DNA molecules and is able to introduce superhelical turns into relaxed circular DNA in the presence of a topoisomerase I activity.

MATERIALS AND METHODS

Materials

DEAE cellulose (DE52) and phosphocellulose (P11) were obtained from Whatman. DNA cellulose was prepared using native calf thymus DNA according to the procedure of Litman (7). The hybrid plasmids containing the whole D-loop region (pXlmB14) or the light strand origin of replication (pXlmB3) were isolated as described in (8). pXlmE2 corresponds to pUC18 with the Eco RI-Hpa I fragment (nucleotides 2089 - 3995 according to Dunon-Bluteau *et al.* (9)) which include the promoters and the O_H replication origin. pXlmH5 corresponds to pUC18 with the Hpa I fragment (nucleotides 520-2088) which contains the region surrounding the 3' end of the D-loop. [³H]poly(dG-dC) and [³H]poly(dG-BrdC) were a gift from Dr. B. Malfoy (Orléans, France). *X. laevis* mtRNA was provided by N. Dennebouy.

Isolation of the mitochondrial DNA binding protein

Mitochondria from *Xenopus laevis* oocytes were purified on a linear sucrose gradient as described in (5). The outer membrane was removed by digitonin according to the procedure of G. Brun *et al.* (10). Purified mitoplasts (~500 mg of proteins) were lysed with 1 % Triton X100 in 20 mM Tris pH 7.5, 1 M NaCl, 2 mM dithioerythritol (DTE), 0.1 mM phenylmethylsulphonyl fluoride (PMSF) at a protein concentration of about 10 mg/ml. The lysate was centrifuged at 200 000 g for 165 min, the clear supernatant was dialysed against 20 mM KPO₄ pH 7.5, 2 mM 2-mercaptoethanol, 20 % glycerol and applied on a DEAE cellulose column equilibrated with the same buffer. Adsorbed proteins were eluted with a linear KPO₄ gradient from 20 to 500 mM. The binding activity was measured by filter binding assays as previously described (8) using an *in vivo* labelled supercoiled DNA. The corresponding fractions were pooled and dialysed against 20 mM Tris pH 7.5, 1 mM EDTA, 2 mM 2-mercaptoethanol, 200 mM NaCl (buffer A 200), 20 % glycerol and loaded on a DNA cellulose column. The column was eluted stepwise with 550 mM and 1 M NaCl. The DNA binding activity was recovered in the 1 M eluate. The fractions were dialysed against 20 mM Tris pH 7.5, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 50 mM NaCl, 60 % glycerol and kept at -20°C.

Except when mentioned in the text the filter binding assays were performed in 200 μ l of the following standard buffer 20 mM KPO_4 pH 5.7, 1 mM EDTA, 2 mM 2-mercaptoethanol, 500 mM NaCl, 5 % glycerol.

Sedimentation analysis

The protein solution in 60 % glycerol was diluted in the standard buffer, immediately layered over a 10 to 30 % glycerol gradient performed in the same buffer and centrifuged at 53000 rpm in a Beckman VTi 65 rotor at 3°C for 3 hours. The fractions were collected and their binding activity measured. The markers BSA and horse cytochrome c were run in a parallel gradient and their distribution was determined spectrophotometrically at 280 nm and 410 nm respectively.

When the preformed DNA-protein complex was run on a glycerol gradient the centrifugation was performed at 49000 rpm in a Beckman SW60 rotor for 2 hours.

Protein gel electrophoresis

Proteins were electrophoresed on an 11 % polyacrylamide slab gel containing 0.1 % NaDod SO_4 using a discontinuous buffer system (11). Protein bands were visualized using the silver staining method (12). The protein amount was estimated by comparison of the intensity of the band with those of markers.

DNA protein association and agarose gel electrophoresis

The topoisomerase I activity was purified from oocyte cytosol according to the procedure of E. Mattocia et al. (13). Relaxed pBR328 was obtained by incubation of the supercoiled form with topoisomerase I in buffer A 200, 5 % glycerol, for 90 min at 30°C. The DNA was then extracted with phenol-chloroform-isoamyl alcohol, then chloroform-isoamyl alcohol and precipitated with isopropanol in the presence of 0.5 M NaCl. The precipitate was washed, dried and dissolved in 10 mM Tris pH 7.5, 1 mM EDTA. 200 ng of this relaxed DNA were incubated in buffer A 200 with topoisomerase I and various amounts of the protein for 90 min at 30°C ; the reaction was stopped by ethanol precipitation. The precipitated DNA was then redissolved in 1% SDS in order to separate it from proteins and analysed by electrophoresis on 1 % agarose gel in 36 mM Tris, 30 mM NaH_2PO_4 , 1 mM EDTA pH 7.7.

RESULTS

Isolation of the protein

The cleared mitoplast lysate was chromatographed on a DEAE cellulose column, the eluted fractions were assayed for their binding activity to supercoiled DNA at 150 mM NaCl (fig. 1). While most of the proteins adsorbed

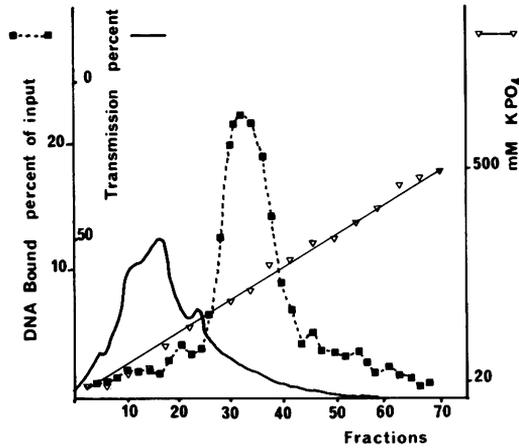


Figure 1 - DEAE cellulose chromatography.

Proteins adsorbed on the column were eluted with a linear 20-500 mM KPO₄ gradient 10 µl of fractions (6 ml) were used to measure the binding activity at 150 mM NaCl with 1.6 µg of supercoiled ³H pBR328.

on the column were eluted at a rather low ionic strength, those showing the ability to bind supercoiled DNA were eluted at ~ 230 mM K Phosphate (or ~ 270 mM NaCl) ; the retention on filters of heat denatured DNA by the same fractions was very low. No binding activity for supercoiled DNA was found in the flow-through fraction. The fractions of the peak were then adsorbed on a DNA cellulose-column, the corresponding activity was eluted at 1M NaCl. The electrophoretic pattern is shown on fig. 2, a major band is found at 31 Kd and two faint ones at 28 and 26 Kd. The addition to the purification procedure of a phosphocellulose column (from which the activity was eluted at ~ 1M KCl) after the DEAE cellulose column and a heparin ultrogel column following the DNA cellulose step did not modify significantly the electrophoretic pattern. Whatever the purification scheme the 31 Kd band was present and usually much more important than the 28 and 26 Kd ones ; however in some preparations the relative ratio was different, the 28 Kd band being the major one. The incubation of the purified fraction did not change the initial ratio. The 28 and 26 Kd polypeptides could be degradation products of the 31 Kd protein, the critical step being, perhaps, the lysis in spite of the presence of PMSF.

From the binding activity in the DEAE peak and assuming that one molecule of protein allows the retention on a filter of one molecule of DNA the amount of protein has been estimated: a minimum of 100 molecules per mtDNA molecule would be present in the mitochondria.

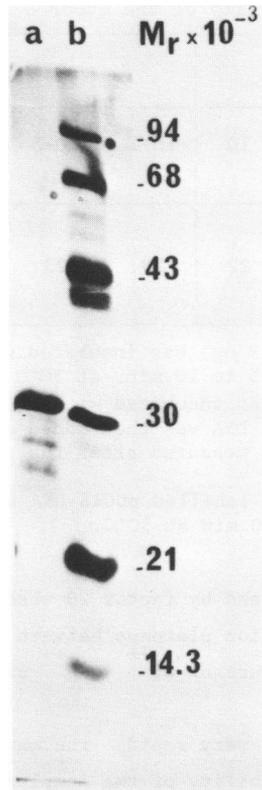


Figure 2 - Na Dod SO_4 polyacrylamide gel electrophoresis pattern of mtDBP C purified by chromatography on DEAE and DNA cellulose column.

lane a mtDBP C

lane b markers (phosphorylase B ; BSA ; ovalbumin ; carbonic anhydrase ; soybean trypsin inhibitor ; lysozyme).

Enzymatic activities were looked for at the different levels of the purification scheme ; no ATPase, RNA or DNA polymerase, topoisomerase I or II activity could be detected associated with the fractions containing the DNA-binding protein.

This protein will be referred as mtDBP C.

In vitro DNA binding properties

The binding of mtDBP C to DNA was measured using the filter binding assay. At 500 mM NaCl the retention is maximum at pH 5.7, its drops down progressively by two thirds when pH reaches 8.6. At pH 5.7 the binding depends sharply on the NaCl concentration, it is reduced by factor 4 when the

Table 1 : Stability of the mtDBP C - DNA complex.

	binding ^a				Dissociation ^b				Control ^c
Time (min.)	0.5	1	2	10	10+1	10+5	10+10	10+60	10
DNA bound (percent of input)	21	20	19	22	20	23	18	21	1

- a) Supercoiled ³H-pUC18 (0.9 µg) was incubated with a non-saturating amount of protein for 0.5 to 10 min. at 30°C.
- b) Supercoiled ³H-pUC18 was incubated with mtDBP C for 10 min ; A ten-fold excess of cold DNA was then added and the labelled DNA retained on a filter was measured after the additional incubation time.
- c) In the control cold and labelled pUC18 DNA were added at the same time and incubated for 10 min at 30°C.

salt concentration is 100 mM and by factor 20 when it is 1 M. On the contrary at pH 7.9 the optimum retention plateaus between 200 and 500 mM NaCl. The addition to the assay mixture of Ca⁺⁺, Mg⁺⁺ or ATP has no effect on the binding efficiency.

The binding reaction is very rapid, the maximum retention is obtained within half a minute. The stability of the complex was determined by studying the fate of the preformed ³H DNA-protein complex after the addition of a large excess (10 fold) of unlabelled homologous DNA : no significative dissociation could be found after an hour as judged by the filter binding assay (Table 1).

Glycerol gradient analysis

From glycerol gradients performed at 500 mM NaCl pH 5.7 a sedimentation coefficient of approximately 2S was found. In such gradients the protein was monitored by its binding activity. Fractions corresponding to the peak of activity as well as those where no activity was found were pooled separately and analyzed on an SDS polyacrylamide gel. The 31 Kd polypeptide and the minor 28 and 26 Kd ones were only found in the first pool. Moreover the three polypeptides were recovered in their initial relative amount. It should be added that when a preformed ³H DNA-protein complex was run on a glycerol gradient only the fractions of the radioactive peak contained polypeptides, their molecular weights were 31, 28, 26 Kd and the bands were in the same ratio as in the initial protein fraction. These results show that the three

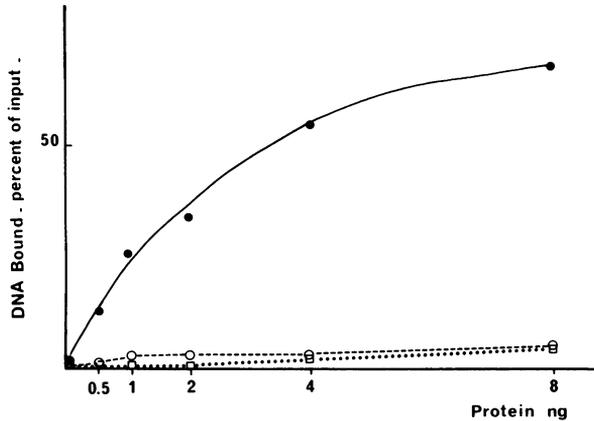


Figure 3 - Affinity for different forms of DNA.

DNA binding activity was assayed in the standard buffer with 300 ng of supercoiled ●—●, linearized □—□ and heat-denatured ○—○ ³H pBR328 DNA.

polypeptides do not correspond to different subunits of a single protein. Either they are three different DNA-binding proteins with similar chromatographic and binding properties, or the 28 and 26 Kd polypeptides are degradation products of the 31 Kd one.

Differential affinity of the protein for various DNAs

Figure 3 shows that the supercoiled form of the pBR328 DNA was retained on a filter (in the standard conditions) with a higher efficiency than the double or single-strand linearized form. This preference for supercoiled DNA molecules was confirmed by competition experiments, (fig. 4-I). pBR328 is about 10 times more effective as a competitor in its supercoiled form than in linearized double or single strand form. The relative affinity of mtDBP C for supercoiled plasmids with or without fragments of mitochondrial DNA overlapping either the O_H (pXlmb14) or the O_L (pXlmb3) origin of replication was very similar indicating that it does not show sequence specificity for these regions, fig. 4-I. It should be noticed that in these experiments the control DNA was the plasmid in its dimeric form to reduce a possible effect due to the size of the plasmids. When the D-loop region was subcloned in shorter fragments and inserted in the pUC18 plasmid no sequence specificity was found (fig. 4-II); this figure also shows that mtDBP C does not bind efficiently to mitochondrial RNAs.

It has been shown (14) that supercoiled DNA molecules having a natural

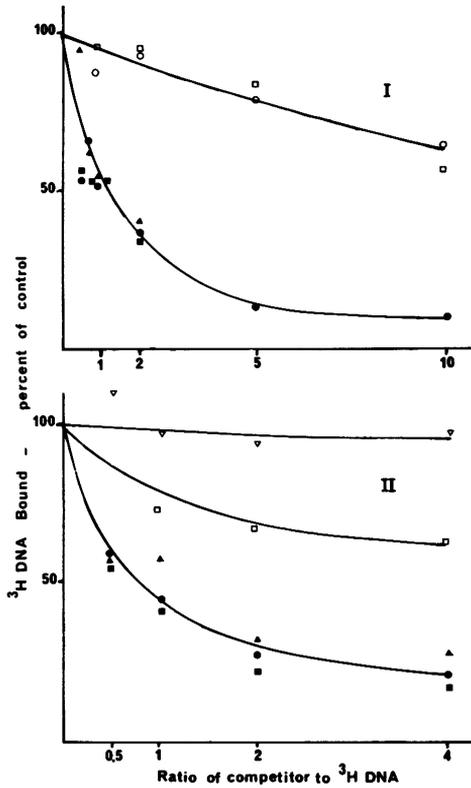


Figure 4 - Affinity for various DNAs and RNA.

I - DNA binding activity was measured with supercoiled ³H pBR328 in the presence of increasing amounts of competitive unlabelled DNAs : linear pBR328 □—□ , denatured pBR328 ○—○ , supercoiled pBR328 ●—● , supercoiled pXlmB14 ■—■ , supercoiled pXlmB3 ▲—▲ .

II - DNA binding activity was assayed with supercoiled ³H pUC18 in the presence of total mitochondrial *X. laevis* RNA ▽—▽ , linear pUC18 □—□ , supercoiled pXlmH5 ▲—▲ , supercoiled pUC18 ●—● , supercoiled pXlmE2 ▲—▲ .

Results are expressed as % of DNA bound in the absence of competitor.

negative superhelical density can contain sequences in the Z form under physiological conditions. Such left-handed segment could be found in mitochondrial DNA and consequently one could anticipate a mitochondrial protein showing a specific binding for Z DNA. In this respect mtDPB C was tested for its binding to Z DNA poly(dG-BrdC) by comparison with the B form poly(dG-dC). Figure 5 shows that it binds to both forms with the same efficiency at 200 mM NaCl. A similar result was obtained at other ionic strengths in the range 50 to 300 mM.

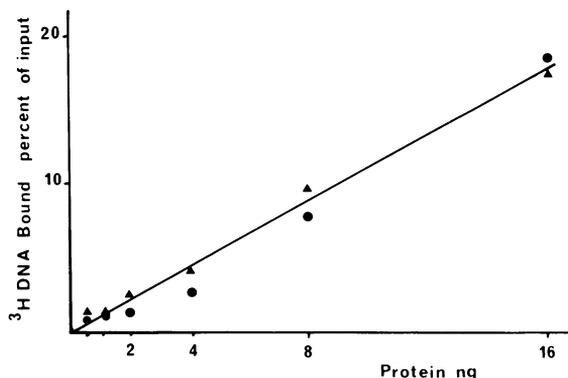


Figure 5 - Affinity for the B and Z forms of a polynucleotide.

Binding activity was measured at 200 mM NaCl, pH 7.5 with 100 pg of either [^3H]poly(dG-dC) (B form) \blacktriangle - \blacktriangle or [^3H]poly(dG-5BrdC) (Z form) \bullet - \bullet in the presence of 200 ng of unlabelled *E. coli* DNA as a competitor for unspecific binding.

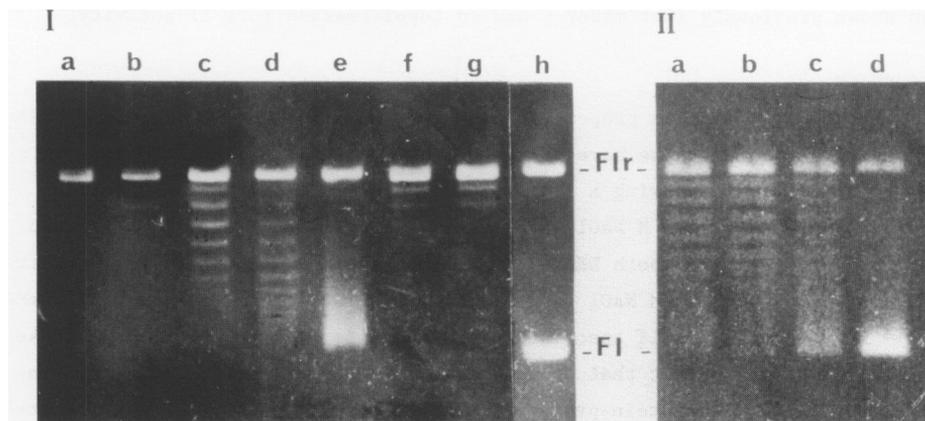


Figure 6 - Modification of the superhelicity of a DNA by mtDBP C in the presence of topoisomerase I.

I - Generation of superhelical turns.

200 ng of purified relaxed pBR328 were incubated with increasing amounts of protein in the presence of purified *X. laevis* topoisomerase I at 200 mM NaCl at 30°C for 90 min. DNA was then analyzed by agarose gel electrophoresis (see material and methods). Lanes a to e correspond to protein-DNA association in the presence of topoisomerase I, the wt/wt protein to DNA ratio was 0.0, 0.1, 0.2, 0.4, 0.8 - lane f. relaxed pBR328 - lane g. same as e without topoisomerase I - lane h. native pBR328.

II. Inhibition of topoisomerase I activity.

200 ng of supercoiled pBR328 were incubated with *X. laevis* topoisomerase I in the presence of increasing amounts of mtDBP C at 200 mM NaCl at 30°C for 90 min. Lanes a to d correspond to protein/DNA ratio of 0.0, 0.02, 0.05 and 0.1.

DNA protein association

If mtDBP C participates in the beaded structure of the nucleoids one could imagine that by its association to relaxed circular DNA, it would introduce negative superhelical turns in the molecule as histones do in nucleosomes. The superhelical turns can be measured in the presence of the topoisomerase I. The purified relaxed pBR328 DNA was incubated with the protein at different protein-DNA ratios in the presence of topoisomerase I for 90 min and analysed on an agarose gel after deproteinisation (fig. 6-I). The number of superhelical turns increases with the amount of protein added reaching a maximum at an approximate weight ratio of 1 (lane e). The DNA still present at the position of form II and relaxed form I (FIR) molecules corresponds to the nicked molecules which were present in the initial DNA preparation (compare lanes e and h). Furthermore the presence of mtDBP C protects supercoiled DNA against the action of the topoisomerase I, fig. 6-II. Both these results show that the association of this protein with covalently closed DNA induces a topological constraint in the molecule. It had been shown previously that mtDBP C had no topoisomerase I or II activity.

DISCUSSION

Using as a selective property a preferential binding to supercoiled DNA at high ionic strength we have isolated from oocyte mitochondrial extracts a DNA binding protein showing a sedimentation coefficient of 2S measured on a glycerol gradient in 0.5 M NaCl which is consistent with a monomer of 31 Kd. The protein adsorbs to both DEAE and phosphocellulose columns from which it is eluted at 0.25 and 1 M NaCl respectively. At low ionic strength (< 200 mM NaCl) it undergoes self association and tends to form aggregates. These properties could suggest that it contains distinct domains involved in binding to DNA and in protein-protein interactions (preliminary experiments indicate a cooperative binding).

The high salt concentration (1 M NaCl) necessary to elute the protein from a DNA cellulose column and an optimum ionic strength of 0.5 M NaCl in the binding assays suggest a rather strong affinity for DNA. The affinity of mtDBP C for supercoiled DNA appear higher than for linear or double-stranded DNA. It was tempting to assign some role to this protein in the specific recognition of supercoiled DNA containing the D-loop region we have previously found in mitochondrial extracts (8). The lack of specific affinity for any DNA sequence precludes the sole involvement of mtDBP C in this preferential binding.

The association of mtDBP C with relaxed circular DNA (FIR) introduces

superhelical turns in the molecules in the presence of topoisomerase I, the maximum was reached for an approximate weight ratio of 1. It also protects supercoiled DNA against the action of topoisomerase I. In this way this protein compares to the yeast HM protein, however no cross reaction of the HM antibodies with mtDBP C has been found (C. Jacq, personal communication). The amount of mtDBP C necessary to convert FIR DNA into FI is about 8 times higher than the one necessary to protect a same amount of form I DNA against the activity of topoisomerase I. Assuming the affinity of mtDBP C is the same for any non-constrained molecule (linear (FIII) or FIR) this reflects the higher affinity (~ 10 times) of mtDBP C for supercoiled DNA. This effect on the superhelicity of a DNA suggests that mtDBP C could be a component of the beaded structures of the nucleoid.

From studies done in the absence of protease inhibitor one acid soluble protein of 28 Kd seemed to be a specific component of nucleoid structures (3). It might be suggested that this polypeptide is the degraded form of the 31 Kd protein characterized in this paper. Antibodies raised against the isolated mtDBP C will be of interest to determine whether the 28 Kd polypeptide of the nucleoid and the 28 Kd degradation product of mtDBP C are identical.

In conclusion we have isolated a mitochondrial DBP which shows the properties expected for the (one of the) structural protein(s) of the nucleoid.

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