Protoplasma (2002) 219: 51–58

PROTOPLASMA

Identification of the YMN-1 antigen protein and biochemical analyses of protein components in the mitochondrial nucleoid fraction of the yeast *Saccharomyces cerevisiae*

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Received June 18, 2001
Accepted September 1, 2001

Summary. We analyzed the protein components contained in the mitochondrial nucleoid (mt-nucleoid) fraction of the yeast *Saccharomyces cerevisiae*. Immunoblotting with anti-Abf2p antibody demonstrated the association of Abf2p, a major mitochondrial DNA-binding protein, with the mt-nucleoids. In contrast, porin and cytochrome *c* oxidase subunit III (CoxIIIp) were not detected by immunoblotting in the mt-nucleoid fraction. The YMN-1 monoclonal antibody recognized a 48 kDa protein of the mt-nucleoid fraction. The N-terminal amino acid sequence of the protein and immunological evidence showed that the YMN-1 monoclonal antibody recognizes dihydrolipoyl transsuccinylase (KE2), which is one of the constituents of the α-ketoglutarate dehydrogenase complex (KGDC). α-Ketoglutarate dehydrogenase (KE1) and dihydrolipoyl dehydrogenase (E3), which are other subunits of KGDC, were also detected in the mt-nucleoid fraction. An enzyme assay of the mt-nucleoid fraction showed that cytochrome *c* oxidase and fumarase activity were barely detected in the fraction, but the specific activity of KGDC in the mt-nucleoid fraction was relatively high and was approximately 60% of the specific activity in the mitochondrial fraction. Three components of KGDC were detected in the DNA-binding protein fractions after DNA-cellulose column chromatography of mt-nucleoid proteins. These results suggested that a part of KGDC in the mitochondrial matrix is associated with mt-nucleoids in vivo.

Keywords: Yeast; *Saccharomyces cerevisiae*; Mitochondria; Mitochondrial nucleoid; α-Ketoglutarate dehydrogenase complex.

Introduction

Mitochondria are the organelles that include the chemical reaction systems needed to synthesize ATP by oxidative phosphorylation, and each mitochondrion has its own genome in the matrix. Each protein component of the mitochondria is encoded by either nuclear DNA or mitochondrial DNA (mtDNA). The mtDNA is three-dimensionally packaged into large complexes (mt-nucleoids) in association with specific proteins (Kuroiwa 1982). The organization of mt-nucleoids is assumed to affect significantly the metabolism of DNA (mtDNA replication, transcription, recombination and genome segregation). The mt-nucleoids from several animals, fungi, and plants have been isolated and characterized (Van Tuyle and McPherson 1979, Suzuki et al. 1982, Sakai et al. 1998, Fey et al. 1999).

We previously isolated the mt-nucleoids from the yeast *Saccharomyces cerevisiae*, while maintaining their morphological integrity (Miyakawa et al. 1987), and demonstrated that mt-nucleoids exist as chromatin-like structures composed of three to four molecules of mtDNA per mt-nucleoid, together with several RNAs and a number of protein components. Later, we were able to identify six species of DNA-binding proteins, which are thought to be involved in packaging of mtDNA (Miyakawa et al. 1995). A unique histone-like protein of 20 kDa, designated as HM, has been isolated from yeast mitochondria (Caron et al. 1979). This protein has been shown to be a major component of isolated mt-nucleoids (Miyakawa et al. 1987, 1995, 2000, 2001). HM, later designated as Abf2p, binds to 30 bp of mtDNA and functions as an essential component in mtDNA stability (Diffley and Stillman 1991). On the other hand, it

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has been shown that the instability of mtDNA associated with the deletion of Abf2p was suppressed by overexpression of other mitochondrial proteins (Zelenaya-Troitskaya et al. 1995, Kao et al. 1996, Cho et al. 1998). These results strongly suggest that a number of mitochondrial proteins other than Abf2p may be involved in maintaining the stability of mtDNA and nucleoid formation.

The mt-nucleoid fraction that was isolated from the yeast *S. cerevisiae* contains a different subset of proteins from that of the total mitochondrial proteins (Miyakawa et al. 1987, 1995, 2001; Shiiba et al. 1997). However, little is known about what kind of proteins are associated with mt-nucleoids in vivo. In order to solve this problem, each protein component that is associated with the isolated mt-nucleoids must be characterized. For this purpose, we previously prepared monoclonal antibodies (mAbs) against the isolated mt-nucleoids and obtained the YMN-1 mAb that recognized a 48 kDa protein of the mt-nucleoid fraction (Miyakawa et al. 1993). In this study, we identified the antigen protein of the YMN-1 mAb as dihydrolipoyl transsuccinylase (KE2) and analyzed the protein components of the mt-nucleoid fraction by immunoblotting and enzyme assay.

Material and methods

Preparation of yeast mt-nucleoids

The diploid strain G2-2 of *Saccharomyces cerevisiae* was cultured aerobically at 30 °C to the stationary phase, as described previously (Miyakawa et al. 1984, 1988). Mitochondria and mt-nucleoids were prepared by the methods described previously (Miyakawa et al. 1987). Cells were converted to spheroplasts by treatment with Zymolyase 20T (Seikagaku Kogyo Co., Ltd., Tokyo, Japan). Mitochondria were prepared from disrupted spheroplasts by differential centrifugation. After lysis of the mitochondria in 0.5% (v/v) Nonidet P-40 (NP40), the supernatant was loaded on a 20, 40, 60% (w/v) discontinuous sucrose gradient. Mt-nucleoids were recovered from the boundary between 20% and 40% sucrose.

Immunofluorescence microscopy

The YMN-1 mAb (immunoglobulin G) was purified from the ascitic fluid by protein A-column chromatography and was used at 1 : 50 dilution. Immunofluorescence microscopy was performed following the methods of Pringle et al. (1991). Spheroplasts were fixed with 3.7% (w/v) formaldehyde for 1 h at room temperature, applied to polylysine-coated coverslips, and fixed for 6 min in methanol and 30 s in acetone at –20 °C. After being washed with phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin (PBS-BSA), the samples were incubated with YMN-1 mAb for 1 h at 30 °C. After a second washing with PBS-BSA, rhodamine-conjugated secondary antibody raised against mouse immunoglobulin G (Tago Co., Burlingame, Calif., U.S.A.) was added at 1 : 40 dilution. After incubation for 1 h at 30 °C, coverslips were washed with PBS and mounted in mounting medium supplemented with 4′,6-diamidino-2-phenylindole (DAPI) (0.25 μg/ml). The isolated mt-nucleoids were fixed with 2% (w/v) paraformaldehyde in 0.3 M sucrose and 10 mM sodium cacodylate buffer, pH 7.4, for 1 h at room temperature, and then immobilized on polylysine-coated coverslips. Methanol and acetone fixations were omitted for the isolated mt-nucleoids. The specimens on slides were observed with an epifluorescence microscope (BH2-RFK; Olympus, Tokyo, Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15% gels by the method of Laemmli (1970). Gels were stained by the silver-staining technique (Oakely et al. 1980). The method used for immunoblotting was based on the method of Towbin et al. (1979). Antibodies that reacted with the antigens on polyvinylidene difluoride filters were detected by the immunoperoxidase procedure using the Vectastain ABC kit (Vector Laboratories Inc., Burlingame, Calif., U.S.A.) as indicated in the instruction manual. Monoclonal antibodies against porin and cytochrome *c* oxidase subunit III (CoxIIIp) (Molecular Probes, Eugene, Oreg., U.S.A.) were used at 1 : 1000 and 1 : 500, respectively. Rabbit antisera against yeast KE1-TrpE and KE2-TrpE fusion proteins were gifts from Dr. A. Tzagoloff (Columbia University) (Repetto and Tzagoloff 1990). Anti-Abf2p polyclonal antibody was raised against purified protein with rabbits (Miyakawa et al. 2000). A low-molecular-weight calibration kit from Pharmacia was used for molecular-weight markers.

Immunoaffinity column chromatography

The YMN-1 mAb-conjugated column assay was performed as described previously (Miyakawa et al. 1993). The supernatant of lysed mitochondria after fractionation of mt-nucleoids was loaded on a YMN-1 mAb-conjugated column. The column was washed with an extensive volume of 0.35 M NaCl in PBS. Adsorbed proteins were eluted with 0.1 M acetic acid that contained 1% NP40. The fraction containing the eluted proteins was used for immunoblot analyses.

Two-dimensional gel electrophoresis

The two-dimensional gel electrophoresis was carried out according to the method of Oh-ishi et al. (1998). Isolated mitochondria and mt-nucleoids were suspended in the sample buffer (5 M urea, 1 M thiourea, 20 mM 2-mercaptoethanol, 2% NP40) and sonicated on ice. After centrifugation at 27,000 **g** for 30 min at 4 °C, the supernatants were applied on the acidic side of the isoelectric focusing agarose gel for the first-dimension electrophoresis. The second-dimension SDS-PAGE was performed on 10% polyacrylamide gel by the method of Laemmli (1970). The gels after the second-dimension electrophoresis were silver-stained or subjected to immunoblotting as described above.

Analysis of amino acid sequence

Proteins from mitochondrial or mt-nucleoid fractions were separated with the two-dimensional gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was stained with amido black. Each spot of the protein was excised, and the N-terminal amino acid sequence of the protein was determined with an Applied Biosystems Procise sequencer.

Enzyme assays

The α-ketoglutarate dehydrogenase complex (KGDC) activity was assayed spectrophotometrically by measuring NAD^+ reduction at 340 nm in a reaction mixture of 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM unacetylated coenzyme A, 1 mM cysteine-HCl, 0.2 mM thiamine pyrophosphate, 0.5 mM NAD^+, 2 mM α-ketoglutaric acid, and 1 mM $MgCl_2$ at 30 °C (Repetto and Tzagoloff 1990). Fumarase activity was assayed by measuring the change of absorption at 250 nm wavelength in a reaction mixture of 50 mM L-malic acid and 90 mM potassium phosphate buffer, pH 7.4 (Kanarek and Hill 1964). Cytochrome *c* oxidase activity was assayed spectrophotometrically by measuring the oxidation of reduced cytochrome *c* at 550 nm in a reaction mixture of 100 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 0.3 mM reduced cytochrome *c* (Tolbert 1974).

We used the YMN-1 immunoglobulin G fraction for the inhibition of enzyme activity. Protein concentration was determined by the method of Lowry (1951) with bovine serum albumin as a standard.

Chromatography on DNA-cellulose

The separation of mt-nucleoid proteins by DNA-cellulose column chromatography was performed as described previously (Miyakawa et al. 1995). The pellet of mt-nucleoids was suspended in 350 µl of TAN buffer (0.5 M sucrose, 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 7 mM 2-mercaptoethanol, 0.4 mM phenylmethylsulfonyl fluoride). 200 U of DNase I (type DSV; Worthington Corp., Freehold, N.J., U.S.A.), 10 mM $MgCl_2$ (final concentration), and 0.4 mM phenylmethylsulfonyl fluoride (final concentration) were added to the suspension and incubated at 25 °C for 30 min. The suspension was then mixed with 350 µl of TAN buffer containing 4 M NaCl and incubated at 25 °C for 30 min. The suspension was centrifuged at 27,000 **g** for 30 min and the supernatant was dialyzed against TAN buffer overnight at 4 °C. After dialysis, the sample was once centrifuged at 27,000 **g** for 30 min and the supernatant was applied to a column of native DNA-cellulose (calf thymus; Pharmacia, Uppsala, Sweden). After the column had been washed with TAN buffer, the bound proteins were eluted with TAN buffer containing 0.2 M NaCl and then with TAN buffer containing 2 M NaCl.

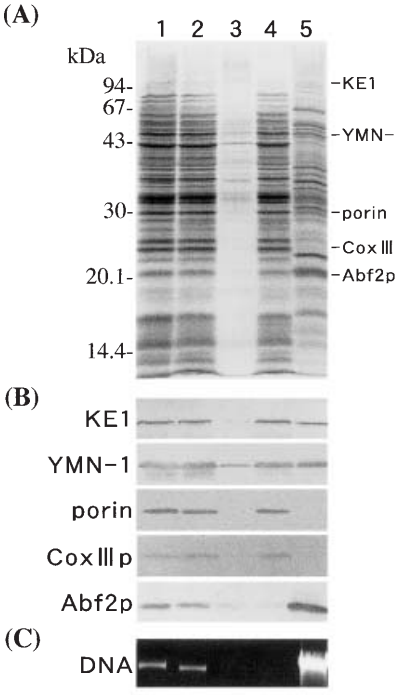

Fig. 1 A–C. SDS-PAGE and immunoblotting of various fractions during the isolation of mt-nucleoids. **A** Aliquots (15 µl) of various fractions during the isolation of mt-nucleoids were loaded on 15% SDS-PAGE and were silver-stained. *1* Mitochondrial fraction; *2* supernatant after lysis of mitochondria by NP40 and subsequent centrifugation (16,000 **g** for 10 min); *3* pellet after lysis of mitochondria and centrifugation; *4* NP40-soluble fraction after discontinuous sucrose-density centrifugation; *5* mt-nucleoid fractions from the boundary between 20% and 40% sucrose. **B** Proteins on the gel were transferred to polyvinylidene fluoride membranes and analyzed for the presence of KE1 (α-ketoglutarate dehydrogenase), YMN-1 antigen (KE2), porin, CoxIIIp, and Abf2p by immunoblotting. The position of each protein is indicated at the right of the gel of **A**. **C** Aliquots (10 µl) of various fractions during the isolation of mt-nucleoids were treated with proteinase K (150 µg/ml) in 0.6% SDS, 12 mM EDTA, 15 mM Tris-HCl, pH 8.0, and 15 mM NaCl at 37 °C for 1 h. The samples were loaded on an agarose gel and stained with ethidium bromide

Results

Localization of the YMN-1 antigen protein

Immunoblot analyses were performed on each fraction during the mt-nucleoid isolation (Fig. 1). Immunoblotting with antiporin and anti-CoxIIIp antibodies revealed that almost all these proteins remained in the supernatant after fractionation of mt-nucleoids and were not detected in the mt-nucleoid fractions (Fig. 1 B). In contrast, Abf2p was highly concentrated in the mt-nucleoid fraction, coinciding with the sedimentation of mtDNA in this fraction (Fig. 1 C). These results show that the degree of contamination by the mitochondrial outer and inner membranes is negligible in mt-nucleoid fractions. On the other hand, the 48 kDa protein which was recognized by the YMN-1 mAb was detected in a considerable amount in the NP40-soluble fraction after fractionation of mt-nucleoids (Fig. 1 B, lane 4) as well as in the mt-nucleoid fraction (Fig. 1 B, lane 5). The volume of mitochondrial and NP40-soluble fractions was 80 ml each, and that of mt-nucleoids was 300 µl. Accordingly, the result indicated that the bulk of the 48 kDa protein remained in the supernatant after fractionation of the mt-nucleoids.

As noted in a previous report (Miyakawa et al. 1993), the YMN-1 mAb labeled many areas in the cytoplasm that corresponded to mt-nucleoids in spheroplasts (Fig. 2 A, B). However, the fluorescence images with the YMN-1 mAb somewhat appeared

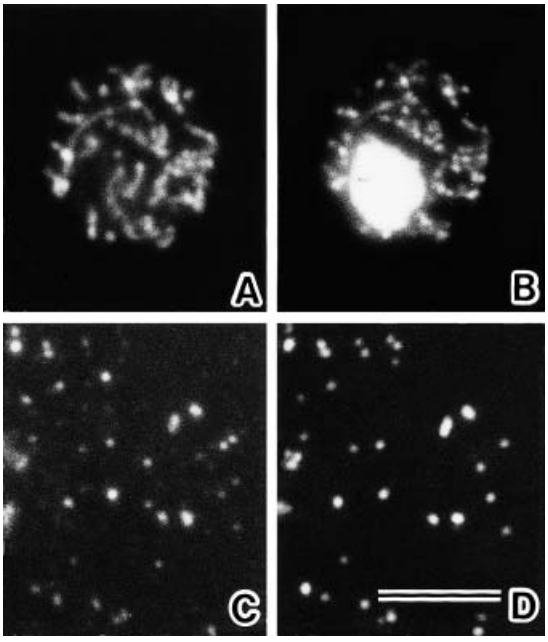

Fig. 2 A–D. Immunofluorescence localization of the YMN-1 antigen. **A** and **B** Spheroplasts of stationary-phase cells were labeled with the YMN-1 mAb and rhodamine-conjugated second antibody for localization of YMN-1 antigen (**A**) and with DAPI for staining of the cell nucleus and mt-nucleoids (**B**). **C** and **D** Isolated mt-nucleoids were also labeled with the YMN-1 mAb (**C**) and stained with DAPI (**D**). The pairs A, B and C, D each show identical fields. Bar: 5 μm

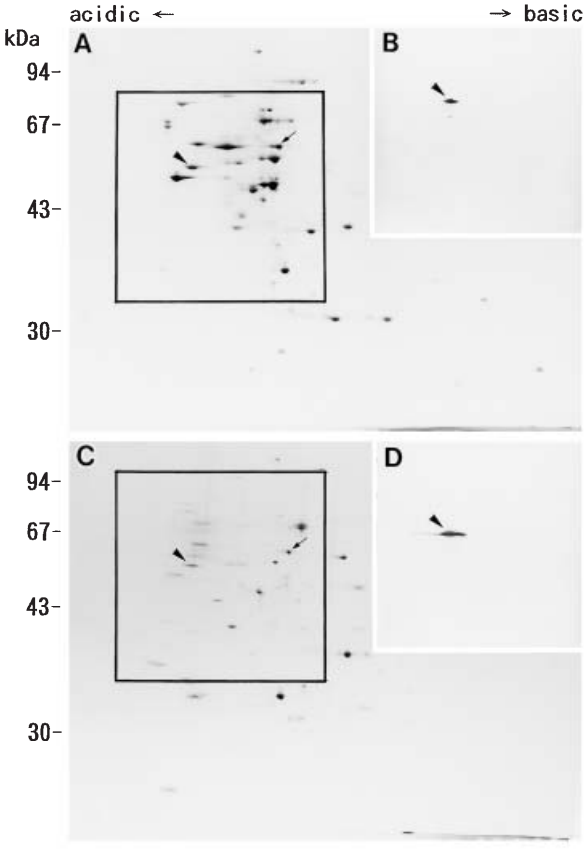

Fig. 3 A–D. Two-dimensional gel electrophoresis and immunoblotting. **A** and **C** Mitochondrial (**A**) and mt-nucleoid proteins (**C**) were analyzed by electrophoresis and silver-stained. **B** and **D** Results of immunoblotting of proteins in the areas boxed **A** and **C**. Arrowheads and arrows indicate the positions of KE2 and E3, respectively

as continuous threads, whereas mt-nucleoids were stained as discrete dots with DAPI. The isolated mt-nucleoids maintained their spherical shapes during fixation with paraformaldehyde (Fig. 2 C, D). The mt-nucleoids stained with DAPI coincided well with particles labeled with the YMN-1 mAb.

Identification of the YMN-1 antigen protein

In order to separate the 48 kDa protein, we analyzed mitochondrial and mt-nucleoid proteins by two-dimensional gel electrophoresis. With this method, a large amount of protein (ca. 500 μg) was loaded on an agarose isoelectric-focusing gel, and proteins ranging from pI 5 to pI 11 were separated, as shown in Fig. 3 A, C. The separated proteins were transferred to a polyvinylidene difluoride membrane and immunoblotted (Fig. 3 B, D). As the YMN-1 mAb recognized a single spot of 48 kDa protein with pI 5.5 in both mitochondria and mt-nucleoids, we determined the N-terminal amino acid sequence of the corresponding spots of mt-nucleoids. The N-terminal 15-amino-acid sequence was KSTSIEVPPMAESLT, and a homology search with the PIR database revealed that this sequence coincided with the sequence from the 72nd to the 86th position of dihydrolipoyl transsuccinylase (KE2), which is a subunit of the KGDC (Repetto and Tzagoloff 1990).

When an NP40-soluble fraction of mitochondria was loaded on a YMN-1 mAb-conjugated immunoaffinity column, the 48 kDa protein was adsorbed to and eluted from the column (Fig. 4, lanes 1 and 2). This protein was intensely recognized by the YMN-1 mAb (Fig. 4, lanes 3 and 4). The anti-KE2-TrpE polyclonal antibody also recognized the same 48 kDa protein (Fig. 4, lanes 5 and 6). These results showed that the YMN-1 antigen is the KE2 subunit of the KGDC.

Additional evidence that KE2 is an antigen of the YMN-1 mAb was obtained from the inhibition of

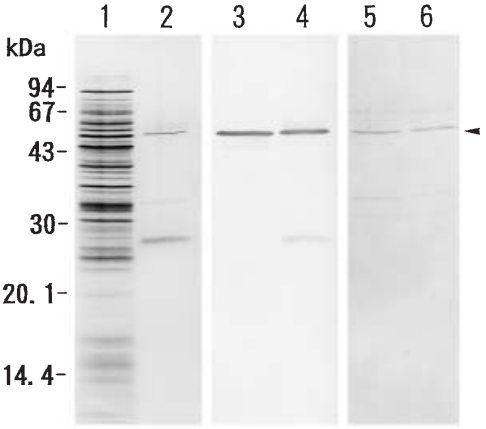

Fig. 4. Immunoaffinity chromatography of the YMN-1 antigen. The NP40-soluble fractions of mitochondria were used to separate the YMN-1 antigen with a YMN-1 mAb-conjugated immunoaffinity column. *1* NP40-soluble fraction; *2* fraction that was adsorbed to and eluted from an immunoaffinity column. *1* and *2* Silver staining after SDS-PAGE; *3* and *4* immunoblotting of lanes 1 and 2 with YMN-1 mAb; *5* and *6* immunoblotting of lanes 1 and 2 with anti-KE2-TrpE polyclonal antibody

Table 1. Inhibition of KGDC activity by YMN-1 mAb [a]

Sample	Specific activity	
	KGDC [b]	fumarase [c]
Without antibody	0.36 (100) [d]	1.31 (100)
With 1 µl of antibody	0.06 (16.7)	1.26 (96.2)
With 2 µl of antibody	0.06 (16.7)	1.26 (96.2)

[a] α-Ketoglutarate dehydrogenase complex (KGDC) and fumarase activity were measured as described in Material and methods. Each sample (40 µl), which contained 40 µg of mitochondrial proteins, was preincubated on ice for 30 min without or with 1 µl (0.7 µg of protein) or 2 µl of the YMN-1 antibody (1.4 µg protein) before initiating the reaction. The reaction was started by adding 20 µl of the sample for KGDC activity or 10 µl of the sample for fumarase activity into a 1 ml reaction mixture
[b] Micromoles of NAD^+ reduced per minute per milligram of protein
[c] Micromoles of fumarate produced per minute per milligram of protein
[d] In parentheses, percent values

KGDC activity by the addition of antibody. The addition and preincubation of the YMN-1 mAb to the mitochondrial fractions prior to the enzyme reaction inhibited KGDC activity by more than 80% (Table 1). The addition of more YMN-1 antibody did not facilitate the inhibition of the enzyme activity. The addition of the antibody had no effect on fumarase activity, another mitochondrial enzyme that is located in the matrix.

Association of KGDC with mt-nucleoids

The N-terminal amino acid sequence of a 50 kDa protein on two-dimensional gel (Fig. 3) was also determined. The sequence was TINKSHDVV, which coincided with the N-terminal sequence of the mature form of dihydrolipoyl dehydrogenase (E3), another subunit of the KGDC (Branda and Isaya 1995). α-Ketoglutarate dehydrogenase (KE1) is an another subunit of KGDC. An anti-KE1-TrpE polyclonal antibody was used for immunoblotting of each fraction of the mt-nucleoid isolation (Fig. 1 B). The KE1 protein with a molecular mass of 110 kDa was detected in the mt-nucleoid fraction as well as other mitochondrial fractions by the antibody. The distribution of KE1 was similar to that of KE2 recognized by the YMN-1 mAb.

Table 2 shows the protein yields and KGDC, fumarase, and cytochrome *c* oxidase activities of the mitochondria and the mt-nucleoid fraction. A part of mitochondrial proteins (ca. 0.8%) was recovered in the mt-nucleoid fraction. Specific activity of fumarase in the mt-nucleoid fraction was only one-eighteenth of that in the mitochondrial fraction. Cytochrome *c* oxidase activity was hardly detected in the mt-nucleoid fraction, which coincided with the absence of CoxIIIp as revealed by immunoblotting (Fig. 1 B). On the other hand, the specific activity of KGDC was high in the mt-nucleoid fraction, relative to that of fumarase and cytochrome *c* oxidase, and was at approximately 60% of the specific activity in the mitochondrial fraction. The yield of total activity of KGDC in the mt-nucleoid fraction was 0.47% of the total mitochondrial activity.

To investigate whether the KGDC has DNA-binding activity, mt-nucleoids were disassembled with DNase I digestion and the following treatment with 2 M NaCl. Then, the proteins that were released

Table 2. Enzyme activity of the mt-nucleoid fraction

Sample	Protein (µg)	Specific activity		
		fumarase [a]	cytochrome *c* oxidase [b]	KGDC [c]
Mitochondria	26200	1.82	1.50	0.35
Mt-nucleoids	207	0.10	0.01	0.21

[a] Micromoles of fumarate produced per minute per milligram of protein
[b] Micromoles of cytochrome *c* oxidized per minute per milligram of protein
[c] Micromoles of NAD^+ reduced per minute per milligram of protein

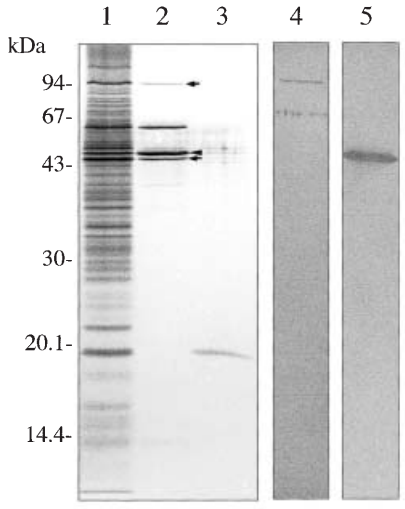

Fig. 5. Chromatography on DNA-cellulose of mt-nucleoid proteins. *1* Isolated mt-nucleoids; *2* a fraction eluted from the DNA-cellulose column with 0.2 M NaCl; *3* a fraction eluted from the DNA-cellulose column with 2 M NaCl after elution of the column with 0.2 M NaCl; *4* immunoblotting of lane 2 with anti-KE1-TrpE antibody; *5* immunoblotting of lane 2 with YMN-1 mAb. The arrows at lane 2 indicate the positions of KE1 and KE2, the arrowhead indicates the position of E3

from mt-nucleoids were applied to a DNA-cellulose column. Four proteins with molecular masses of 110 kDa, 67 kDa, 50 kDa, and 48 kDa were mainly eluted with 0.2 M NaCl (Fig. 5, lanes 1 and 2). Among them, the 110 kDa and 48 kDa proteins were recognized as KE1 and KE2 by immunoblotting with the anti-KE1 antibody and the YMN-1 mAb, respectively (Fig. 5, lanes 2, 4, and 5). The N-terminal sequence of the 50 kDa protein coincided with that of E3 (Fig. 5, lane 2). The 20 kDa protein Abf2p was eluted as a homogeneous band with 2 M NaCl from the column (Fig. 5, lane 3).

Discussion

In an attempt to identify and localize the protein components associated with mt-nucleoids, we immunized mice with isolated mt-nucleoids and obtained the YMN-1 mAb which intensely recognized a 48 kDa protein of the mt-nucleoids. We expected that the YMN-1 antigen would be localized exclusively to the mt-nucleoids, but the 48 kDa protein was abundantly present in the NP40-soluble fraction of mitochondria as well. In the present study, we demonstrated that the antigen protein for the YMN-1 mAb is the KE2 component of the KGDC. This conclusion was bolstered by immunological evidence using antibody against the KE2-TrpE fusion protein and from the specific inhibition of KGDC activity of mitochondria by the YMN-1 mAb (Figs. 1–4 and Table 1). The precursor form of KE2 has 463 amino acids (Repetto and Tzagoloff 1990). In this study, the N-terminal amino acid sequence of KE2 shows that the N-terminal 71-amino-acid residue is a mitochondrial targeting signal and is cleaved to form the mature enzyme. Since the yeast KE2 is about 70 residues longer than the *E. coli* protein at the N-terminal end (Repetto and Tzagoloff 1990), cleavage of the N-terminal 71 residue by mitochondrial protease coincides well with the predicted mature form of KE2. Besides KE2, we identified on a two-dimensional gel a 50 kDa protein in the mt-nucleoid fraction as the E3 subunit of KGDC (Figs. 3 and 5). Furthermore, immunoblotting with an anti-KE1-TrpE antibody also revealed the KE1 component (110 kDa) in the mt-nucleoid fractions (Figs. 1 and 5). Together with the results of the enzyme assay, these results showed that the KGDC, although representing only a small fraction of total mitochondrial activity, cosedimented with mt-nucleoids during sucrose-density fractionation of the mt-nucleoids from lysed mitochondria.

The results of this study do not indicate that mt-nucleoid fractions are heavily contaminated with mitochondrial membranes and matrix enzymes that are not involved in mt-nucleoid organization. Newman et al. (1996) modified our methods for isolating mt-nucleoids and reported that the mt-nucleoid fraction was contaminated with mitochondrial membrane proteins, as revealed by immunoblot analysis of porin and CoxIIIp. However, as shown in Fig. 1, neither porin nor CoxIIIp was detected by immunoblotting of mt-nucleoids, whereas Abf2p and mtDNA are highly concentrated in mt-nucleoid fractions (Fig. 1). The enzyme assay of fumarase and cytochrome *c* oxidase activity also showed a very low level of contamination by matrix and inner-membrane proteins (Table 2). The discrepancy of our results and those of Newman et al. (1996) may result from the difference of protein concentration of mitochondria at the lysis of mitochondrial membrane with NP40, as Newman et al. (1996) suspended the isolated mitochondria at 20-fold the protein concentration of our method. We confirmed with an antiporin antibody that an increase of the protein concentration of mitochondria at the lysis of mitochondria with NP40 causes an insufficient solubilization of mitochondrial membranes and the

contamination of the mt-nucleoid fraction with the mitochondrial membrane.

Recently, Kaufman et al. (2000) developed an in organello formaldehyde cross-linking procedure to identify proteins associated with mtDNA. After the treatment of isolated mitochondria with formaldehyde, mitochondria were lysed and applied directly to a CsCl gradient. Those authors identified 11 proteins cross-linked to mtDNA. These proteins included Abf2p, Rim1p, and Mgm101p, which have been shown to bind mtDNA in vitro and in vivo. In addition, Kgd2p (KE2 subunit) and Lpd1p (E3 subunit) were also identified among them. Especially, Kgd2p (KE2) was recovered in the cross-linked material at nearly the same level as Abf2p. Furthermore, genetic experiments demonstrated that the *kgd2*Δ mutation increased the petite-inducing phenotype of an *abf2*Δ mutation, indicating the direct participation of Kgd2p in the stability of mtDNA.

In the present study, we first demonstrated that three components of the KGDC (KE1, KE2, and E3) are associated with isolated mt-nucleoids and were recovered in the DNA-binding protein fractions after dissassembly of mt-nucleoids and the DNA-cellulose chromatography. A previous study suggested that the YMN-1 antigen protein, which was separated from mt-nucleoids by immunoaffinity chromatography, has the ability to bind to mt-nucleoids in vitro (Miyakawa et al. 1993). However, the isolated mt-nucleoids tended to aggregate during formaldehyde fixation and the intensity of immunofluorescence in isolated mt-nucleoids was lower than that in spheroplast and in isolated mitochondria with the YMN-1 mAb (Miyakawa et al. 1993). In the present study, the mt-nucleoids that were fixed with paraformaldehyde were distinctly labeled with the YMN-1 mAb, without the formation of large aggregates of mt-nucleoids during the fixation.

The KGDC has an essential role in the tricarboxylic acid cycle in the mitochondrial matrix and catalyzes the oxidative decarboxylation reaction of α-ketoglutarate to succinyl coenzyme A (Reed 1974, Yeaman 1989). The core structure of this macromolecular enzyme (molecular weight, 2.5×10^6 to 2.8×10^6) consists of multiple copies of KE2 to which dimers of KE1 and E3 are noncovalently bound (Repetto and Tzagoloff 1991). At present, little is known how the KGDC binds to mtDNA and how the macromolecular enzyme is involved in three-dimensional folding of mtDNA. Analyses of interaction between the purified enzymes and mtDNA will solve the possible roles of the KGDC in mt-nucleoid formation.

The mtDNA instability phenotype of cells with a null allele of the *ABF2* gene (*abf2*Δ) was also suppressed by overexpression of Ilv5p, acetohydroxy acid reductoisomerase, which catalyzed a step in branched-chain amino acid biosynthesis (Zelenaya-Troitskaya et al. 1995). Among the cross-linked proteins, Ilv5p was also shown to associate with mtDNA (Kaufmann et al. 2000). These results suggest that some matrix enzymes as well as the KGDC also participate in the organization of mt-nucleoids in addition to their enzymatic activity. Therefore, we are continuing to identify each mt-nucleoid protein separated by means of two-dimensional gel electrophoresis.

Acknowledgments

We thank Dr. A. Tzagoloff (Columbia University) for the generous gift of yeast KE2-TrpE and KE1-TrpE polyclonal antibodies and Dr. M. Oh-ishi (Kitazato University) for technical advice on two-dimensional gel electrophoresis. This work was supported in part by grants to I.M. (nr. 09680697) from the Ministry of Education, Science and Culture of Japan.

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