AN ADP- AND ATRACTYLOSIDE-BINDING PROTEIN INVOLVED IN ADP/ATP TRANSPORT IN YEAST MITOCHONDRIA

Isolation and amino acid composition

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The adenine nucleotide carrier protein from yeast mitochondria has been purified by extraction with Triton X-100 of the complex made by the carrier protein with carboxyatractyloside followed by chromatography on SP Sephadex and QAE Sephadex. The purified protein gives a single band on SDS–polyacrylamide gel electrophoresis corresponding to mol. wt 37 000. The amino acid composition indicates the predominance of hydrophobic amino acids. The polarity index [5] is 39%.

1. Introduction

Two procedures are used at present to purify the adenine nucleotide carrier protein from mammalian mitochondria. The first one is based on the ability of the carrier protein to firmly bind the specific inhibitors, atractyloside and carboxyatractyloside and on the principle of affinity chromatography using succinyl atractyloside or succinyl carboxyatractyloside [1,2] bound to Sepharose. The second procedure takes advantage of the fact that the adenine nucleotide carrier is one of the few mitochondrial proteins which is not retained by a hydroxyapatite gel [3].

In [4] it was shown that photo-irradiation of mitochondria from *Saccharomyces cerevisiae* in the presence of photo-activable derivatives of atractyloside or ADP resulted in the photo-labeling of a 37 000 mol. wt protein. This protein therefore belongs to the adenine nucleotide transport system and is referred to here as the mitochondrial adenine nucleotide carrier protein. The purification and the amino acid composition of this protein is now described. The purification procedure involves an extraction by Triton X-100 of the complex made by the carrier protein with carboxyatractyloside followed by chromatography on SP Sephadex and QAE Sephadex. The purified protein gives a single band on SDS–polyacrylamide gel electrophoresis corresponding to mol. wt 37 000. The amino acid composition indicates the predominance of hydrophobic amino acids. The polarity index [5] is 39%.

Abbreviations: SP Sephadex, sulfopropyl Sephadex; QAE Sephadex, quaternary amino ethyl Sephadex; Mops, morpholinopropane sulfonic acid; DTT, dithiothreitol; EDTA, ethylene diamine tetracetic acid; PMSF, phenyl methyl sulfonyl fluoride; DFP, diisopropylfluorophosphoric acid; SDS, sodium dodecyl sulfate

2. Materials and methods

The yeast strain used in this study was a diploid of *S. cerevisiae* JB65 (P9/P9) obtained from Dr J. Mattoon. The yeast mitochondria were prepared by the method in [6]. [^{3}H]Atractyloside and [^{3}H]arylazido atractyloside were prepared as in [1] and [4], respectively. SDS–polyacrylamide gel electrophoresis was carried out as in [7] using a 10.3% acrylamide gel. SP Sephadex and QAE Sephadex were purchased from Pharmacia. Protein was determined with the Folin-Ciocalteu reagent [8] as modified [9] for traces of SDS; bovine serum albumin was taken as standard. Amino acid analysis was carried out on the mol. wt 37 000 protein extracted from SDS–polyacrylamide gel after gel electrophoresis. A blank was run under similar conditions, i.e., with an extract of SDS–polyacrylamide gel not loaded with protein. The conditions of gel extraction are detailed in section 3. The composition was determined with a Technicon Autoanalyzer after total hydrolysis (6 M HCl at 110°C for 18 h, 48 h and

72 h under vacuum). The tryptophan content was established after hydrolysis of the protein with methane sulfonic acid [10]. Cysteine was characterized as cysteic acid after performic acid oxidation [11].

3. Results and discussion

3.1. *Purification of the yeast adenine nucleotide carrier protein by ion exchange chromatography*

Step 1: The yeast mitochondria (100 mg protein) were allowed to contact for 15 min at room temperature with 240 nmol carboxyatractyloside in 40 ml of a medium made of 0.63 M mannitol, 0.1 mM DTT, 10 mM Mops (pH 6.8), 0.5 mM EDTA, 0.2 mM PMSF and 5 mM DFP. The suspension was then centrifuged at 20 000 × *g* for 10 min. All subsequent manipulations were conducted at 4°C.

The mitochondrial pellet was resuspended in 30 ml medium made of 6% Triton X-100, 100 mM NaCl, 0.5 mM EDTA, 0.2 mM PMSF and 10 mM Mops (pH 7.4). After 30 min, the mitochondrial lysate was centrifuged at 100 000 × *g* for 90 min to remove membrane fragments. The Triton extract (80–85 mg protein) was recovered and adjusted to pH 6 with diluted phosphoric acid, the volume being finally brought to 40 ml. As carboxyatractyloside binds firmly to the adenine nucleotide carrier in mitochondria at neutral pH [12], the form of the carrier protein which is present in the Triton extract is the carboxyatractyloside-bound form.

Step 2: The solution was passed through a column of SP Sephadex C-50 (13 × 5.5 cm) which had been pre-equilibrated with 30 mM sodium phosphate buffer (pH 6), 100 mM NaCl, 0.1 mM DTT, 0.2 mM PMSF, 0.5 mM EDTA and 0.1% Triton X-100. The pass-through fraction (50 mg protein) corresponding to the void volume was concentrated to 30 ml by pressure filtration using an Amicon PM10 membrane. The pH was adjusted to 9 with 1 M Tris buffer and the fraction was then dialyzed against 3 l distilled water for 3–4 h with two changes till the resistance reaches a value of 400 Ω.cm (measured at 0°C).

Step 3: The dialyzed fraction was poured on a column of QAE Sephadex A-25 (11 × 5.5 cm) pre-equilibrated with 50 mM NaCl, 0.1 mM DTT, 0.1% Triton X-100, 0.5 mM EDTA, 0.2 mM PMSF and 10 mM Tris (pH 9). The pass-through fraction (~13 mg protein) was immediately adjusted to pH 7.2 with diluted phosphoric acid and concentrated to 15 ml by pressure filtration using an Amicon PM10 membrane. It contained essentially two proteins with mol. wt 37 000 and 34 000, as revealed by SDS–polyacrylamide gel electrophoresis (fig.1). This fraction can be kept at –80°C after rapid freezing in liquid nitrogen until the next step. At pH 9, the bound carboxyatractyloside was released to the medium as shown by a parallel purification assay made on a small batch of yeast mitochondria treated by radiolabeled carboxyatractyloside. Therefore the subsequent steps deal with the carboxyatractyloside-free carrier protein.

Step 4: The final step consisted of a second chromatography on SP Sephadex G-25. A column of SP Sephadex G-25 (15 × 2.5 cm) was equilibrated with the same buffer (pH 6.0) as that used for the first SP Sephadex chromatography. The fraction from step 3 was adjusted to pH 6.0 and applied to the SP Sephadex column. The pass-through fraction corresponding to the void volume consisted of the pure mol. wt 34 000 protein. After washing the gel with about 10 ml equilibrium buffer (pH 6.0) another medium made of 200 mM Na-bicarbonate buffer (pH 9), 500 mM NaCl, 0.1 mM DTT, 0.2 mM PMSF, 0.5 mM EDTA and 0.1% Triton X-100 was passed through the SP Sephadex column, and 2 ml fractions were collected. Fractions 10–15 contained the mol. wt 37 000 protein. The purified protein was concentrated by

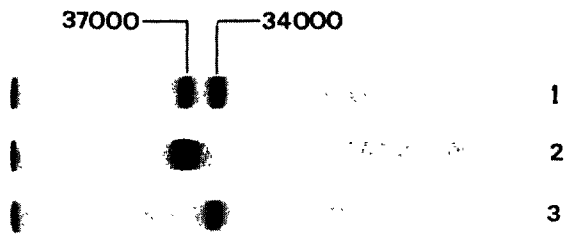

Fig.1. SDS–polyacrylamide gel electrophoresis of fractions obtained by chromatography on SP Sephadex and QAE Sephadex. Gel 1 corresponds to the fraction recovered at step 3. Gels 2 and 3 show the mol. wt 34 000 and 37 000 proteins recovered at step 4.

pressure filtration on Amicon PM10 filter. It gave only one band by SDS–polyacrylamide gel electrophoresis (fig.1).

To summarize, two successive chromatographies on SP Sephadex and QAE Sephadex, followed by rechromatography on SP Sephadex resulted in rapid purification of the mol. wt 37 000 protein.

3.2. *Characterization of the adenine nucleotide carrier protein*

The mol. wt 37 000 protein in *S. cerevisiae* mitochondria was identified as the adenine nucleotide carrier protein by its ability to bind covalently photoactivable derivatives of ADP and atractyloside, namely azidonitrophenyl γ-aminobutyryl ADP and the azidonitrophenyl γ-aminobutyryl atractyloside. These derivatives were prepared in their radiolabeled form; their use to photolabel the adenine nucleotide carrier has been extensively described [4]. When incubated with *S. cerevisiae* mitochondria under light, they were able to bind covalently to only one protein whose mobility on SDS–polyacrylamide gel electrophoresis corresponded to mol. wt 37 000.

3.3. *Failure to purify the yeast adenine nucleotide carrier protein by hydroxyapatite chromatography from a Triton extract*

Hydroxyapatite chromatography has been described as a procedure for rapid purification of the adenine nucleotide carrier protein from beef heart mitochondria. The same method was applied to the purification of the carrier protein from *S. cerevisiae* mitochondria. The extraction of *S. cerevisiae* mitochondria by Triton X-100 was as in section 3.1. The extract was passed through a 5 cm diam. column containing 80 ml decanted hydroxyapatite gel equilibrated with 100 mM NaCl, 0.5% Triton X-100, 10 mM Mops (pH 7.2). The pass-through (10 mg protein) was collected as 2 ml fractions which were concentrated by pressure filtration on Amicon PM10 membrane. SDS–polyacrylamide gel electrophoresis revealed 2 proteins with mol. wt 34 000 and 37 000 in about equal amounts in all fractions collected. Rechromatography on hydroxyapatite failed to separate the 2 proteins.

3.4. *Amino acid composition*

Amino acid analysis was carried out on the mol. wt 37 000 protein extracted from SDS–polyacrylamide gel by either of the above two methods, in order to compare the amino acid composition of the mol. wt 37 000 protein purified by double chromatography on SP and QAE Sephadex and that of the mol. wt 37 000 protein which comigrates with the mol. wt 34 000 protein after hydroxyapatite chromatography. We routinely started from 20–22 gels and 1–1.2 mg protein (0.5 mg/ml) recovered by either of the two purification methods. One-third of the protein preparation (0.4 mg) was treated by 80 μl freshly prepared solution of fluorescamine (4 mM) in dimethylformamide after alkalinisation (pH 9) by addition of a small amount of 200 mM sodium carbonate buffer (pH 9) (fluorescamine permits an easy detection of proteins on polyacrylamide gel after separation by electrophoresis [13]. After 30 s contact, the pH was brought back to neutrality with 1 N HCl. The protein fraction stained by fluorescamine was mixed with the untreated protein fraction, and the protein material was precipitated by addition of 5 vol. acetone at −20°C. The precipitate was dissolved in 1 ml 3% SDS, 30% glycerol, 2% mercaptoethanol, and heated for 2 min at 100°C. Fractions (~50 μg) were applied to SDS–polyacrylamide gels. After electrophoresis in the dark, the gels were observed under ultraviolet light and the fluorescent bands corresponding to mol. wt 37 000 were sliced. The slices were fragmented by passage through a syringe needle, then extracted twice with 10 ml 5 mM phosphate buffer (pH 7.2) at room temperature, for 1 h each time. After centrifugation, the supernatant fluid was concentrated by filtration on PM10 Amicon membrane. The protein recovery was 50–60%. A blank extract was systematically carried out. In fact, polyacrylamide gels contain some impurities which react with ninhydrin and may obscure the amino acid analysis. The blank values were deduced in the amino acid compositions given in table 1. The mol. wt 37 000 proteins separated by gel electrophoresis from material purified by chromatography on SP and QAE Sephadex or by chromatography on hydroxyapatite proved to be similar in their amino acid composition; the small differences are not significant. This can be taken as further indication but not as a proof that the mol. wt 37 000 protein is a homogeneous material. In fact, the adenine nucleotide carrier could be a

Table 1
Amino acid composition of the mitochondrial adenine nucleotide carrier protein from *S. cerevisiae* purified by chromatography on SP and QAE Sephadex (SP-QAE) or by chromatography on hydroxyapatite (HTP)

	SP-QAE[a]	HTP
Asp	31	31–32
Thr	17	17
Ser	24	24
Glu	20	20
Pro	13	13
Gly	35–36	35–36
Ala	37	37–38
Val	24	24–25
(Cys-)	3– 4	4
Met	5– 6	5– 6
Ile	17	17
Leu	36	36
Tyr	13	13
Phe	23	23
Trp	3	n.d.
Lys	23	23
His	4– 5	n.d.
Arg	15	15

[a] Mean values after analysis of 4 different preparations

Residues/mol of assumed mol. wt 37 000: nearest integer established after different hydrolysis times

dimer made of subunits similar by their molecular weight, but differing in their amino acid composition; in that case, the amino acid composition given here would have to be considered as an average value for both subunits. The polarity of the adenine nucleotide carrier protein from yeast calculated as in [5] is 39%, a value very close to the value of 40% reported in [14] for the adenine nucleotide carrier protein of beef heart mitochondria.

References

[1] Brandolin, G., Meyer, C., Defaye, G., Vignais, P. M. and Vignais, P. V. (1974) FEBS Lett. 46, 149–153.
[2] Bojanovski, D., Schlimme, E., Wang, C. S. and Alaupovic, P. (1976) Eur. J. Biochem. 71, 539–548.
[3] Riccio, P., Aquila, H. and Klingenberg, M. (1975) FEBS Lett. 56, 133–138.
[4] Lauquin, G. J. M., Brandolin, G., Lunardi, J. and Vignais, P. V. (1978) Biochim. Biophys. Acta 501, 10–19.
[5] Capaldi, R. A. and Vanderkooi, G. (1972) Proc. Natl. Acad. Sci. USA 69, 930–932.
[6] Balcavage, W. X. and Mattoon, J. R. (1968) Biochim. Biophys. Acta 153, 521–530.
[7] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[8] Zak, B. and Cohen, J. (1961) Clin. Chim. Acta 6, 665–670.
[9] Dulley, J. R. and Grieve, P. A. (1975) J. Biol. Chem. 64, 131–141.
[10] Liu, T.-Y. (1972) Methods Enzymol. 25, 44–55.
[11] Hirs, C. H. W. (1956) J. Biol. Chem. 219, 611–621.
[12] Vignais, P. V., Vignais, P. M. and Defaye, G. (1973) Biochemistry 12, 1508–1519.
[13] Eng, P. R. and Parkes, C. O. (1974) Anal. Biochem. 59, 323–325.
[14] Klingenberg, M., Riccio, P. and Aquila, A. (1978) Biochim. Biophys. Acta 503, 193–210.