Chapter 2

Native Membrane Proteins vs. Yeast Recombinant: An Example: The Mitochondrial ADP/ATP Carrier

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Abstract

The mitochondrial ADP/ATP carrier (Ancp) has long been a paradigm for studies of the mitochondrial carrier family due to, among other properties, its natural abundance and the existence of specific inhibitors, namely, carboxyatractyloside (CATR) and bongkrekic acid (BA), which lock the carrier under distinct and stable conformations. Bovine Anc1p isolated in complex with CATR in the presence of an aminoxyde detergent (LAPAO) was crystallized and its 3D structure determined. It is the first mitochondrial carrier structure resolved at high resolution (2.2 Å, as reported by Pebay-Peyroula et al. (Nature 426:39–44, 2003)). Analyses revealed a monomer while most of the biochemical studies led to hypothesize Ancp functions as a dimer. To address the structural organization issue, we engineered a mutant of the yeast Ancp that corresponds to a covalent homodimer in view of 3D structure determination. We compare in this chapter the purification yield and quality of the chimera tagged either with six histidines at its C-ter end or nine histidines at its N-ter. We show that, as expected, length and position of the tag are important criteria for qualitative purification. We also discuss the advantages and drawbacks of purifying Ancp either from a natural source or from engineered yeast cells.

Key words: Mitochondrial ADP/ATP carrier, Mitochondrial carrier, High yield purification

1. Introduction

Major metabolic pathways occur within mitochondria, which is the primary site of ATP synthesis. It is isolated from the cytosol by two membranes (outer and inner) that are therefore important sites for the regulation of metabolic functions. The mitochondrial carrier family (MCF) members are integral membrane proteins that transport various metabolites through the mitochondrial inner membrane. Among them, the mitochondrial ADP/ATP carrier (Ancp) was the first to be identified around 40 years ago.

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It plays a key role in the energetic cell metabolism because it exchanges ATP and ADP, respectively product and substrate of the mitochondrial ATP synthase. Ancp is the most abundant among MCF members (up to 10% of mitochondrial proteins in beef heart mitochondria) and has to cope with high nucleotide amounts in order to fulfill cell energetic requirements. It can be purified pretty easily in one or two steps from beef mitochondria in high amount and in crystallization compatible quality. However, the high-resolution structure of the beef Anc1p in complex with its inhibitor carboxyatractyloside (CATR) evidenced a monomeric organization (1). This was contradictory to many previously published results about different MCF members, of which Ancp shares functional and structural features and was shown to be organized as dimers (for a review see (2)). We hypothesized that protein preparation, and more precisely, the protein concentration step necessary to crystallization trials was responsible for Ancp dimer dissociation. Therefore, we used an engineered Ancp mutant (3) that forces a dimeric organization of the carrier throughout the purification and crystallization processes; we named it AA in this chapter. However, we previously showed that it was necessary to tag the yeast Ancp to get it highly purified (4). Consequently, the chimera was tagged with either six histidines at its C-ter (AAH6) or nine histidines at its N-ter (H9AA).

2. Materials

2.1. Chemicals

1. *n*-dodecyl-β-D-maltoside (DDM) was purchased from Anatrace. [^{14}C]DDM was kindly provided by Marc Le Maire (CEA, CNRS, Université Paris-Sud 11, France).
2. Atractyloside (ATR) and carboxyatractyloside (CATR) were obtained from Sigma.
3. The nickel-nitrilotriacetic acid agarose matrix (Ni-NTA) for metal affinity chromatography is from Qiagen. It is provided as a 1:1 (vol/vol) suspension in 50% ethanol (vol/vol).
4. 3-Laurylamido-*N*,*N*′-dimethylpropylaminoxide (LAPAO) was synthesized as described in (5). It can now be purchased from Anatrace.
5. Hydroxyapatite Bio-Gel® was purchased from Bio-Rad.
6. Thrombin was from Sigma and used as recommended by the supplier.

2.2. Buffer Compositions

1. Buffer A: 150 mM NaPi pH 7.3, 10% (w/v) glycerol, 0.1% (w/v) DDM.

2. Buffer B: 100 mM Na_2SO_4, 10 mM Tris–HCl pH 7.3 and 1 mM Na_2-EDTA pH 8.0.
3. Buffer C: 33% (v/v) glycerol, 6 mM $MgSO_4$, 150 mM NaPi pH 7.3.
4. Buffer D: 150 mM NaPi pH 7.3, 10% (w/v) glycerol, 0.05% (w/v) DDM, 40 mM imidazole pH 8.0.
5. Buffer E: 10 mM MOPS-NaOH pH 6.8, 150 mM NaCl, 10% (w/v) glycerol, 0.05% (w/v) DDM.
6. Buffer F: 500 mM imidazole pH 8.0, 10 mM MOPS-NaOH pH 6.8, 150 mM NaCl, 10% glycerol, 0.05% (w/v) DDM.
7. Buffer G: 10 mM MOPS-NaOH pH 6.8, 150 mM NaCl, 10% glycerol, 0.05% (w/v) DDM.
8. Buffer H: 100 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM Na_2-EDTA, 0.05% LAPAO (w/v).
9. Buffer I: 500 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM Na_2-EDTA.
10. Buffer J: 10 mM Tris–HCl pH 7.4, 1 mM Na_2-EDTA, 0.05% LAPAO.

3. Methods

3.1. The 6-Histidine Tag: Mutagenesis, Plasmids, and Strains

1. Construction of the gene encoding Anc2p tagged with six histidines at its C-ter (AH6) is described in (4).
2. To construct the gene coding for the covalent tandem dimer of Anc2p tagged with six histidines at its C-ter (AAH6), the wild type *ScANC2* gene was amplified with the following primers: 5′-GAATTCGGATCCATGTCTTCCAACGCCC AAGTCAAAA-3′ and 5′-CTGGATCCTTTGAACTTCTTA CCAAACAAGATC-3′ to introduce *Bam*HI sites (underlined) on each side of the ORF and remove the stop codon. The fragment was then subcloned into the unique *Bam*HI site of the pAH6 plasmid (4), which contains the gene encoding for AH6. After control of the orientation of the fragment, the 3′ *ScANC2* terminator region (amplified with two primers containing each an *Xba*I site) was introduced. The resulting (*Pst*I-*Not*I) fragment containing the promoter, the AAH6 encoding gene and the terminator was used for its integration into the JL1-3Δ2 strain (6) by homologous recombination at the *ANC2* locus. The resulting strain was named JL-AAH6.

3.2. The 9-Histidine Tag: Mutagenesis, Plasmids, and Strains

1. The *His9(ANC2)*$_2$ gene was obtained by site directed mutagenesis using the Transformer™ Site-directed Mutagenesis Kit (CLONTECH Laboratories). The protein produced from

this gene corresponds to a covalent tandem homodimer of Anc2p (3) tagged with nine histidines at its N-ter.

2. The mutagenesis target plasmid was obtained from KSDIM5′3′ (3) that was digested first by *Sal*I and *Not*I to shorten the *ScANC2* 3′ noncoding region. The resulting plasmid was named KSDIM5′3′ΔSN.
3. The mutagenic primer 5′catacataaagcaaatacaattgccATG GGT **CAC CAT CAC CAC CAT CAC CAT CAC** CAC TCT TCA GGT TTA GTT CCT AGA GGT *TCT TCC AAC GCC CAA G3′* was designed (1) to replace the single *Eco*RI site of KSDIM5′3′ΔSN with a *Mfe*I site (lower case, underlined) to select for mutagenesis events; (2) to introduce a cluster of nine histidines (capital, bold) at the N-terminus of the covalent tandem dimer of Anc2p (capital, italic); and (3) to introduce the recognition and digestion sites of thrombin (capital, underlined). Successful mutagenesis was assessed by DNA sequencing. The resulting plasmid was named KSH9DIM.
4. The *His9ANC2* gene was obtained by digestion of KSH9DIM by *Hin*dIII and re-ligation of the biggest fragment to obtain a single copy of the $ScANC_2$ gene. The resulting plasmid was named KSH9MON.
5. The *Kpn*I-*Sac*I fragment containing either the *His9ANC2* or the $His9(ANC2)_2$ gene was used for its integration into the JL1-3Δ2 strain (6) by homologous recombination at the *ANC2* locus. The resulting strain was named JL-H9A or JL-H9AA, and the produced protein is H9A or H9AA.

3.3. Isolation of Mitochondria from Yeast

1. Protocol and materials used to perform mitochondria isolation are described in (6). They are frozen in small beads in liquid nitrogen and stored at –70°C prior to protein purification (see Note 1).
2. In the case of H9AA and AAH6, a cocktail of protease inhibitors (1 µg/mL pepstatin A, 1 µg/mL leupeptin, 1 µg/mL antipain, 5 µg/mL aprotinin, and 1 mM Na_2-EDTA, final concentrations) is added to the buffers during mitochondria isolation to prevent protein degradation (7).

3.4. Isolation of Mitochondria from Bovine Heart

Bovine mitochondria were isolated from heart muscle by differential centrifugation as described by (8). They were suspended in 0.27 M sucrose, 2 mM Tris–HCl pH 7.4 and stored in liquid nitrogen (see Note 1).

3.5. AAH6 Purification

1. The protocol used to purify AH6 and described in (4) is applied to purify AAH6. It consists of three steps: hydroxyapatite Bio-Gel® chromatography, immobilized metal ion chromatography, and removal of imidazole by dialysis or AcA202 chromatography.

3.6. H9A and H9AA Purifications

1. The Ni-NTA resin (Qiagen) (1 ml resin for 5 mg total protein) is washed twice with 5 vol H_2O then equilibrated with Buffer A (2 × 3 resin volumes).
2. When necessary, isolated mitochondria (100 mg) are incubated in the presence of 600 nmol CATR for 15 min at 4°C.
3. Membrane proteins are solubilized with 1% (w/v) or 3% (w/v) DDM in Buffer B for 15 min at 4°C supplemented with an antiprotease cocktail as described in (7) in the case of H9AA. During this step the final protein concentration is 10 mg/mL.
4. The lysate is centrifuged at 24,000 × *g* for 10 min at 4°C and the supernatant is supplemented with Buffer C (0,5 vol for 1 vol of supernatant) and 20 mM imidazole. It is loaded onto the equilibrated Ni-NTA resin using a batch procedure and incubated for 1 h at 4°C in a rotating apparatus.
5. The flow-through fraction is removed by a 10-min centrifugation at 4,000 × *g* and 4°C.
6. The resin is then resuspended with two volumes of Buffer D and poured into a column. It is thereafter washed with two volumes of the same buffer. The two eluted fractions (four resin volumes) are pooled for further analyses.
7. The resin is washed with five volumes of Buffer E. The eluate is usually collected in two fractions: one corresponds to the first 4.5 × resin volumes and the other to the last 0.5 × resin volume.
8. The protein is eluted with three resin volumes of Buffer F. Elution of the protein is followed by absorbance measurement at 280 nm.
9. Imidazole is removed by an Ultrogel® AcA 202 size exclusion chromatography (four resin volumes for one sample volume). The eluant is Buffer G. Elution of the protein is followed by absorbancy measurement at 280 nm. Both H9A and H9AA proteins are obtained highly purified as shown in Fig. 1.

3.7. Protein Concentration

1. The protein concentration after Ultrogel® AcA 202 chromatography is far from being sufficient for crystallization trials. The fraction of interest is loaded on Amicon® Centriprep® YM30 units (Millipore). The molecular weight cutoff is 30,000 Da. The units are centrifuged at 4°C and 900 × *g* to reduce ten times the fraction volume.
2. Detergent is concentrated at the same time and this may lead to protein denaturation. Thus, detergent is removed using by Bio-Beads® SM2.
3. Bio-Beads are activated as described in (10).

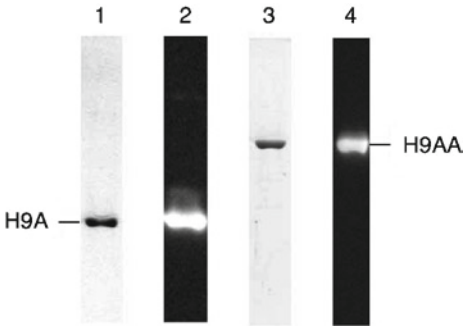

Fig. 1. Analyses of purified H9A and H9AA. Ten microliters of the imidazole elution fraction (*lanes 1 and 2*: H9A; *lanes 3 and 4*: H9AA) is analyzed by SDS-PAGE (12.5%). The gels are divided in two parts, one is Coomassie blue stained (*lanes 1 and 3*) and the other is transferred onto a nitrocellulose membrane (*lanes 2 and 4*) and immunostained with an antibody directed against a peptide corresponding to the last 14 amino acids of ScAnc2p as described in (17).

4. The amount of Bio-Beads necessary to remove 90% of the detergent is calculated considering that 100 mg (dry weight) of Bio-Beads after activation can bind 1.8 mg of DDM after 3 h at 4°C, as determined using [^{14}C]DDM. Therefore, the sample should contain at the end of this step about 0.05% DDM corresponding approximately to the initial DDM concentration.
5. The concentrated protein fraction is incubated with the appropriate Bio-Beads amount for 3 h at 4°C under mild shaking (see Note 2). Bio-Beads are removed by three successive centrifugations (3 min, 3,000 × *g*).
6. Steps 4 and 5 are repeated until the Ancp concentration reaches around 10 mg/mL. Protein concentration is determined by absorbance measurement at 280 nm. The molar extinction coefficients are calculated from the H9A and H9AA tryptophanyl and tyrosyl residue content (11): 35,870/M cm for H9A and 71,740/M cm for H9AA.
7. Concentrated H9A and H9AA can be stored at 4°C but preferably are immediately submitted to crystallization trials or to tag removal process.

3.8. Removal of the 9-Histidine Tag

1. Enzyme units (0–20) of thrombin are added to 1 mg of tagged H9AA in 1 ml and left over for 20 h at 4°C with mild shaking. Under such conditions, the histidine tag is almost completely removed for a thrombin to protein ratio of 20 U/mg (Fig. 2).

3.9. Purification of bAncp

1. Hydroxyapatite is suspended in an ice-cold Buffer H and washed with the same buffer according to the supplier's instructions.
2. Mitochondria (50 mg of mitochondrial proteins) are unfrozen and incubated in Buffer I (4 ml final volume) with 25 µM CATR for 10–15 min at 0°C.

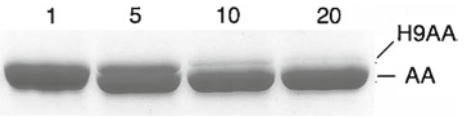

Fig. 2. Polyhistidine tag removal. 0, 5, 10, or 20 enzymatic units of thrombin are added to 1 mg of tagged H9AA in 1 ml and left over for 20 h at 4°C. The samples are analyzed by SDS-PAGE (12.5%), and the protein is revealed by Coomassie blue staining.

3. Membrane proteins are solubilized by addition of 1 mL 10% (w/v) LAPAO and stirring of the mixture. After standing on ice for 10 min, the mitochondrial lysate is centrifuged at 20,000 × *g* for 10 min.
4. The supernatant is layered on a hydroxyapatite Bio-Gel® column (2.5 cm diameter, 25 mL settled gel) and the elution is performed using Buffer H and monitored online by UV absorbance at 280 nm.
5. The pass-through fraction is collected and concentrated to approximately 10 mL by pressure dialysis on an Amicon YM30 membrane. It is then subjected to a gel-exclusion chromatography on Ultrogel AcA202 resin (BioSepra) to remove small solutes such as Ca^{2+} ions, phosphate and nucleotides from the bAncp preparation. In addition, this step allows the protein preparation to be placed under appropriate conditions with respect to salt concentration and pH; whereas equivalent to dialysis, it is considerably shorter and in this way is probably less damaging. In most cases, the chromatography is carried out in a column (2.5 cm diameter) containing 40 ml of settled gel equilibrated in Buffer J, supplemented with either 5 or 100 mM NaCl.
6. The protein fraction containing the purified BAncp·CATR complex is treated with moist activated (see above) Bio-Beads (Bio-Rad, 100 mg per mg protein) for 2 h at 4°C to remove excess detergent (see Note 2). It is then filtered on a 0.45-μm nitrocellulose filter and concentrated to approximately 10 mg/mL on a Centricon YM 30 device (Amicon). In this preparation, the protein is exclusively present as the Anc1p isoform as assessed from in-gel proteolysis combined to mass spectrometry analysis.

3.10. Comments on Use of Native bAncp vs. Recombinant ScAncp

3.10.1. Practical Aspects

Experiments carried out with the bovine ADP/ATP carrier obviously benefit from the abundance and easiness to obtain the amount of biological material required for biochemical investigations. This is first illustrated by the fact that 5–10 g of mitochondrial proteins can be routinely extracted within a few hours from one beef heart. This amount corresponds to almost 0.5–1 g of bAncp. In addition, purification of bAncp is undoubtedly facilitated by the easiness of mitochondria solubilization with detergents

and by the high efficiency of adsorption chromatography on hydroxyapatite (12). This allows recovery of pure carrier in a single step with a 90–100% yield.

Yeast mitochondria are isolated to a lower yield and operation extends over a longer time if considering 1–2 days required for cell growth. Approximately 10–20 mg of mitochondrial proteins, corresponding to 0.5–1 mg of ScAnc2p depending on the nature of strains, are extracted from 1 l of culture.

Attempts to isolate ScAnc2p by chromatography on hydroxyapatite led to preparations containing other mitochondrial proteins such as the phosphate carrier and VDAC in various amounts, depending on the detergent nature. Therefore, it was necessary to engineer polyhistidine-tagged forms of ScAnc2p that we purified by IMAC.

3.10.2. The Genetic Approach

Yet, yeasts offer the undeniable advantage of genetic approaches to investigate structure–function relationships of the ADP/ATP carrier essentially because (1) they contain endogenous Ancps; (2) their genetics is well known; and (3) they are well suited for the rapid screening of the functional state of carrier mutants due to their ability to grow under either fermentative or respiratory conditions. Site-directed mutagenesis has been used to locate strategic amino acid residues expected to play a role in the transport mechanism. Mutated forms of Anc2p able to sustain the growth of yeast on nonfermentable carbon sources were characterized with respect to ADP/ATP transport in isolated mitochondria or in proteoliposomes. Other approaches consisted in removing/introducing appropriate residues in ScAncp without impairing its full transport activity for probing ligand-induced conformational changes of the isolated carrier or for assessing the topography of the membrane embedded carrier. This is illustrated, for example by the use of tryptophanyl mutants using fluorometric approaches (13,14) or that of cysteinyl mutants for chemical labeling experiments (reviewed in (2)).

Genetic handling of yeasts was used with the purpose of functional and/or structural explorations to engineer chimeras in which ScAncp was linked to itself (3,15), to the phosphate carrier (7) or to cytochrome *c* (16), and also for the heterologous expression of the human Ancps to understand the role of pathogenic point mutations (6).

Yeast will undoubtedly afford the means to isolate an Anc2p mutant stabilized in the BA conformation which so far could not be crystallized from isolated beef Ancp due to the difficulties to handle a stable bAncp·BA complex. BA refers to bongkrekic acid, which is the other specific Ancp inhibitor. It is recognized that CATR and BA, the binding of which to Ancp is mutually exclusive, stabilize the ADP/ADP carrier in two distinct conformations. This led to the conclusion that Ancp adopts at least two

different conformations in the membrane, which are probably involved in nucleotide transport. Therefore understanding this process at the molecular level involves deciphering the 3D structure of the ScAnc2p BA complex in comparison with the 3D structure of the Ancp CATR complex.

4. Notes

1. It is recommended to store the frozen mitochondria as 50–100 µL beads. This facilitates the withdrawing of the wanted amount of material prior to each experiment. Beads are made immediately after isolation of mitochondria by dropping the suspension from a pipette into a small volume of liquid nitrogen. They are then handled with a spatula or with forceps.
2. The mixture is gently stirred in a tube rotating horizontally to prevent extensive bead break up.

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