Use of Preparative SDS Gel Electrophoresis Followed by 2-D PAGE for the Purification of a 30 kD Phosphoprotein Involved in the Control of Steroid Hormone Biosynthesis

Elizabeth Griffin Green and Nanette Roberts Orme-Johnson, Dept. of Biochemistry, Tufts University Medical School, Boston, MA 02111 USA

Introduction

This report describes the purification of a low-abundance 30 kD phosphorylated mitochondrial protein using Bio-Rad's Model 491 prep cell, followed by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). A large number of purification methods, including conventional column chromatography of detergent-extracted inner mitochondrial membrane, have not been successful for isolating this protein. The only effective purification method that we have found thus far is 2-D PAGE. However, this procedure has not yielded enough material to obtain an amino acid sequence or to generate antibodies. Additionally, it is very time-consuming and expensive. The Model 491 prep cell has afforded us a much more efficient purification scheme.

Our laboratory is interested in the regulation of steroid hormone biosynthesis by the control of the rate-limiting step, the conversion of cholesterol to pregnenolone. This reaction is catalyzed by the cytochrome P-450 complex located in the inner mitochondrial membrane. We have examined protein synthesis patterns in cells in which these hormones are synthesized, such as the fasciculata of the adrenal cortex, Leydig cells of the testes, and the corpus luteal cells of the ovaries, by analysis of solubilized radiolabeled cells using 2-D PAGE. With this technique (Garrels 1979), we have demonstrated that the addition of stimulant (either trophic peptide hormone or second messenger analog) causes the appearance of a newly synthesized, phosphorylated mitochondrial protein with molecular mass 30 kD (known as pp30). Extensive studies correlating the accumulation of pp30 with the increase in the rate of synthesis of steroid hormones suggest that pp30 may be important in the regulation of acute steroidogenesis (Epstein and Orme-Johnson 1991, Green and Orme-Johnson 1991). Both pp30 and its unphosphorylated form, p30, are integral membrane proteins and thus hydrophobic.

Methods

Sample Preparation

Mitochondria were prepared from rat adrenal glands and mitochondrial inner membrane was isolated by conventional techniques. The inner membrane preparation was extracted with detergent to produce a fraction enriched in p30. The extracted proteins were obtained by harvesting the supernatant resulting from centrifugation at 100,000 x g for 1 hr. The supernatant was concentrated to 20 mg/ml using Amicon stirred ultrafiltration cells with PM10 membranes (10 kD molecular weight cutoff).

Preparative SDS-PAGE

Analytical SDS-PAGE was used to determine the percent acrylamide monomer needed to optimize the separation of 30 kD proteins from proteins of higher molecular weight. This preliminary analysis was performed using the Mini-PROTEAN® II dual slab cell. A 10% acrylamide concentration (%T) was found to be the best choice.

The conditions for the preparative SDS-PAGE were as follows:

Sample	1.0–1.5 ml of detergent extract of rat mitochondria (22–33 mg protein) mixed with an equal amount of Laemmli SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol [w/v], 5% β-mercaptoethanol) was heated to 95°C prior to loading onto the gel
Gel tube	37 mm OD
Gel	10% SDS-polyacrylamide (27.3:2.67 acrylamide: bis-acrylamide, w/w) resolving gel (100 ml) with a 4% stack (10 ml)
Running conditions	10 W constant power, Bio-Rad's Model 1000/500 power supply (equivalent to the current PowerPac™ 1000 power supply)
Elution buffer	SDS running buffer (0.1% SDS, 50 mM Tris, 384 mM glycine)
Elution rate	1.0 ml/min
Fractions	6 ml fractions collected
Length of run	15–17 hr

Results

SDS-PAGE was used to locate fractions containing proteins of the molecular weight of interest in the eluate from the Model 491 prep cell. Beginning with the dye front, 0.5 ml of every fifth fraction was mixed with an equal volume of 20% trichloroacetic acid and incubated for 15 min at 4°C to precipitate the proteins. The samples were centrifuged at 13,000 x g, the pellet was washed in 100% ethanol, and the proteins were solubilized in Laemmli SDS sample buffer containing 5% β-mercaptoethanol. SDS-PAGE was carried out using the Mini-PROTEAN II dual slab cell and the gels were stained with 0.25% Commassie Blue. Figure 1 illustrates the protein content of the fractions collected. The fractions around the 30 kD molecular weight marker were pooled (11 fractions in each run) and concentrated to 3.5 mg/ml using the Amicon stirred ultrafiltration cells Models 3 and 8050 with PM10 membranes. The 30 kD pooled fractions had a total protein content of 3.3 mg. A total of 110 mg of detergent extract was loaded over four different runs. Thus, the overall yield of the 30 kD region is 3%.

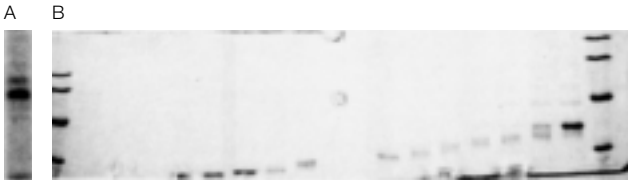

Fig. 1. Separation by preparative SDS gel electrophoresis of a detergent extract of rat adrenal cortex mitochondria. Panel A illustrates the detergent extract prior to treatment on the Model 491 prep cell. Panel B shows two SDS-PAGE gels of fractions obtained after electrophoresis of the crude sample using the Model 491 prep cell. Every fifth fraction after the elution of the dye front from the prep gel was analyzed by removing one-sixth of the fraction and precipitating the protein using TCA. The gels are stained with Coomassie Blue dye. The molecular weight markers seen in the gel on the left and the gel on the right are 97 kD, 66 kD, 43 kD, and 30 kD.

The concentrated pool of Model 491 prep cell fractions containing the 30 kD proteins was analyzed by 2-D PAGE as follows. An aliquot from the pooled Model 491 prep cell fractions, containing 75 µg of protein, was loaded onto the first-dimension isoelectric focusing tube gel (ampholyte ratio 1:2:2 of 3.5–10, 5–7, 6–8), followed by SDS-PAGE. In addition, 75 µg of the crude starting material was analyzed in the same manner. Both gels were silver-stained simultaneously (Figure 2). The Model 491 prep cell afforded a large purification of proteins in the 30 kD region. The protein p30 is visible in the 2-D gel of the enriched fraction (Figure 2B), while it is not detectable in that of the starting material (Figure 2A). This enrichment of the p30 region greatly reduced the number of 2-D gels necessary to purify a sufficient amount of p30 to perform protein sequencing and the production of antibodies.

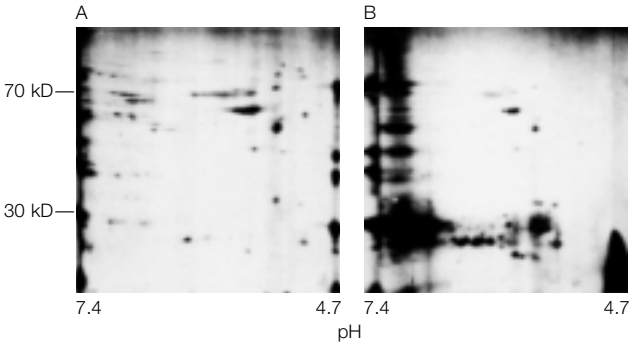

Fig. 2. Two-dimensional gels comparing a detergent extract of rat adrenal cortex mitochondria to 30 kD proteins isolated from the extract by use of the Model 491 prep cell. Panels A and B are silver-stained 2-D gels of the detergent extract, prior to fractionation using the Model 491 prep cell, and of a concentrated pool of the 11 fractions from the Model 491 prep cell containing ~30 kD proteins, respectively. These gels also contain a trace amount of highly radioactive cellular extract which is used to identify the position of p30. Thus, the upper molecular weight contamination in panel B may be due to the presence of this material. The position of migration of molecular weight markers in the SDS dimension is indicated to the left of panel A. The pH in the IEF dimension is indicated below each gel.

References

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