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MICROHETEROGENEITY OF THE GLYCOPROTEIN SUBUNIT OF THE (SODIUM + POTASSIUM)-ACTIVATED ADENOSINE TRIPHOSPHATASE FROM THE ELECTROPLAX OF ELECTROPHORUS ELECTRICUS

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SUMMARY: Isoelectric focusing of purified Na,K-ATPase on polyacrylamide gels resolved the protein into ten bands. The catalytic and glycoprotein subunits were separated by sodium dodecyl sulfate gel filtration. Isoelectric focusing of the isolated glycoprotein subunit showed that it accounted for nine of the ten bands. Part of this microheterogeneity can be attributed to variations in sialic acid content in individual bands, since removal of all of the sialic acid by neuraminidase treatment reduced the number of bands to four. It is suggested that the microheterogeneity of the glycoprotein subunit is due to post-translational modifications of oligosaccharides on a common polypeptide backbone.

Several proteins have now been shown to display microheterogeneity on isoelectric focusing (1-4). These proteins appear to be homogeneous in size and charge on sodium dodecyl sulfate polyacrylamide gel electrophoresis, but on isoelectric focusing, they resolve into multiple bands. Explanations offered for this microheterogeneity have been the presence of multiple structural genes giving rise to different protein species, as well as post-translational modifications such as acylation, phosphorylation, sulfation, and glycosylation (5). However, these post-translational modifications have only been documented in a few instances.

In this preliminary report, we also document microheterogeneity of the glycoprotein subunit of the Na,K-ATPase isolated from the electric organ of Electrophorus electricus. The microheterogeneity can partly be accounted for by variation in the sialic acid content of the oligosaccharide chains.

MATERIALS AND METHODS

The Na,K-ATPase used in these experiments was prepared from electroplax tissue from Electrophorus electricus by the method of Dixon and Hokin (6). The specific activity averaged 1310 μmole/mg protein hr^{-1}.

Abbreviations: SDS, sodium dodecyl sulfate.

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The subunits were isolated from the Na,K–ATPase by gel filtration using a column of Bio-Gel A 1.5m (200-400 mesh). The enzyme (40 mg) was solubilized in 5% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 0.1 M Tris HCl (pH 7.4) with a final volume of 5 ml. It was layered on the Bio-Gel column (2.5 x 80 cm), which had been equilibrated with 1% SDS in 0.1 M Tris HCl (pH 7.4). The protein was eluted with the equilibration buffer. The flow rate was 20 ml/hr, and the eluant was measured at 280 nm using a Gilson 261 dual wavelength UV monitor. The subunit fractions measuring greater than 0.1 OD were collected, pooled, and dialyzed against three changes of 1 mM Tris EDTA (pH 7.4) over a 48 hr period. The subunits were concentrated to a final concentration of 1-2 mg/ml using an Aminco Diaflo PM-10 membrane, and then checked for contamination of the other subunit by SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined by the method of Peterson (7).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out by the method of Laemmle (8). Samples were prepared by adding 50 µl of sample buffer containing 10% glycerol, 5% β-mercaptoethanol, 3% SDS, and 0.06 M Tris HCl (pH 8.3) to 100 µl of sample. One hundred µl of this solution were applied to 8.75% analytical polyacrylamide gels, and the electrophoresis was carried out as described by Hokin et al. (9).

Isoelectric focusing on polyacrylamide gels was carried out by a modification of the O'Farrell technique (10). The gel composition was: 7% acrylamide, 1.9% N,N–methylene–bisacrylamide, 3.5% Nonidet–40, 9 M urea, 2% ampholines (LKB 3–10, 4–6, 5–7), 0.07% N,N,N',N'–tetramethylethylene diamine, and 0.02% ammonium persulfate. The gels were overlaid with 8 M urea for 2 hr, followed by "lysis buffer" (10) for 2 hr, and then pre-electrofocused according to the following schedule: 200 volts for 15 min, 300 volts for 30 min, and 400 volts for 30 min. The samples were heated for 5 min at 100° in 2% SDS, 1% β-mercaptoethanol, and 0.05 M Tris HCl (pH 7.4) in order to insure solubilization. Heating did not produce artifacts since samples prepared without heating produced the same isoelectric focusing pattern. The sample was cooled to room temperature, and 200 µl of "lysis buffer", which contained 9.5 M urea, 8% Nonidet–40, and 2% ampholines, were added. Seventy–five µg of urea were then added, and 50–150 µl of the sample solution were placed on the gel. Twenty–five µl of one–half diluted lysis buffer were layered over the sample. The cathode solution was 0.02 M NaOH, and the anode solution was 0.01 M H_3PO_4. The samples were electrofocused for 4800 volt-hr. They were fixed in 10% trichloroacetic acid with three changes of the trichloroacetic acid solution, and washed once in 25:10:65 ethanol:acetic acid:water. The gels were then stained and destained following the method of Righetti and Drysdale (11). All the solutions were either prepared fresh, using reagent grade chemicals, or stored at –20° prior to use.

Neuraminidase from Clostridium perfringens obtained from Sigma was used to treat the glycoprotein. The incubation solution contained the glycoprotein (2 mg/ml) and neuraminidase (0.04 mg/ml) in 20 mM imidazole HCl (pH 7.0), and it was incubated for 24 hr at 37°. The protein was then precipitated with 5% trichloroacetic acid and centrifuged at 650X g for 10 min. The resulting supernatant was assayed for neuraminidase-sensitive sialic acid by the thiobarbituric acid method of Warren (12). By resuspending the pellet in 0.1 M H_2SO_4 and heating for 2 hr at 80°, the neuraminidase-resistant sialic acid was determined. Total sialic acid was measured following heating of the untreated enzyme samples for 2 hr at 80° with 0.1 M H_2SO_4.

RESULTS AND DISCUSSION

When the Na,K–ATPase from the electroplax was run by SDS gel electrophoresis, the two subunit bands and an additional faint band at the position of the tracking dye

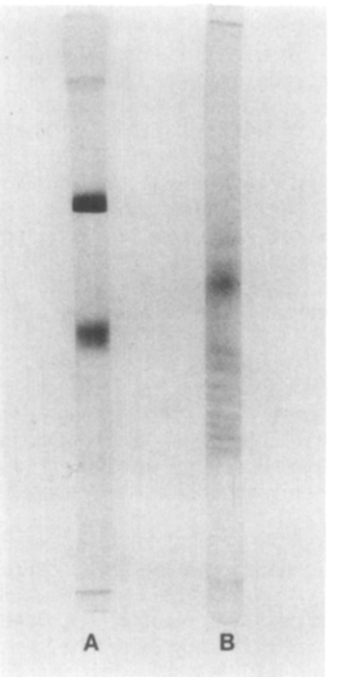

Fig. 1. Electrophoretic behavior of purified Na,K–ATPase from electroplax Na,K–ATPase. (A) sodium dodecyl sulfate polyacrylamide gel electrophoresis of enzyme (75 μg) on 10% gels. (B) isoelectric focusing pattern of enzyme (75 μg) on a polyacrylamide gel composed of 2% ampholines (LKB) 3.5–10, 4–6, and 5–7 in equal proportions.

were seen (Fig. 1A). Only one N–terminal amino acid has been found for each of the purified subunits from the electroplax Na,K–ATPase, attesting to their purity (13). Thus far, alanine has been consistently found as the N–terminal amino acid for the glycoprotein in all species examined (14). However, upon isoelectric focusing in a gradient composed of an equal mixture of LKB ampholytes (pH 3–10, 4–6, 5–7), the enzyme resolved into ten bands (Fig. 1B). When SDS gel electrophoresis was performed in the second dimension, nine of the isoelectric bands corresponded in molecular weight with the glycoprotein (not shown). Because of difficulties in quantitatively solubilizing the catalytic subunit from the isoelectric focusing gel after running in the first dimension, it was decided to separate the two subunits by gel filtration. This technique proved to be effective in quantitatively separating the two subunits as is shown in Fig. 2. Both the chromatogram and the heavily loaded

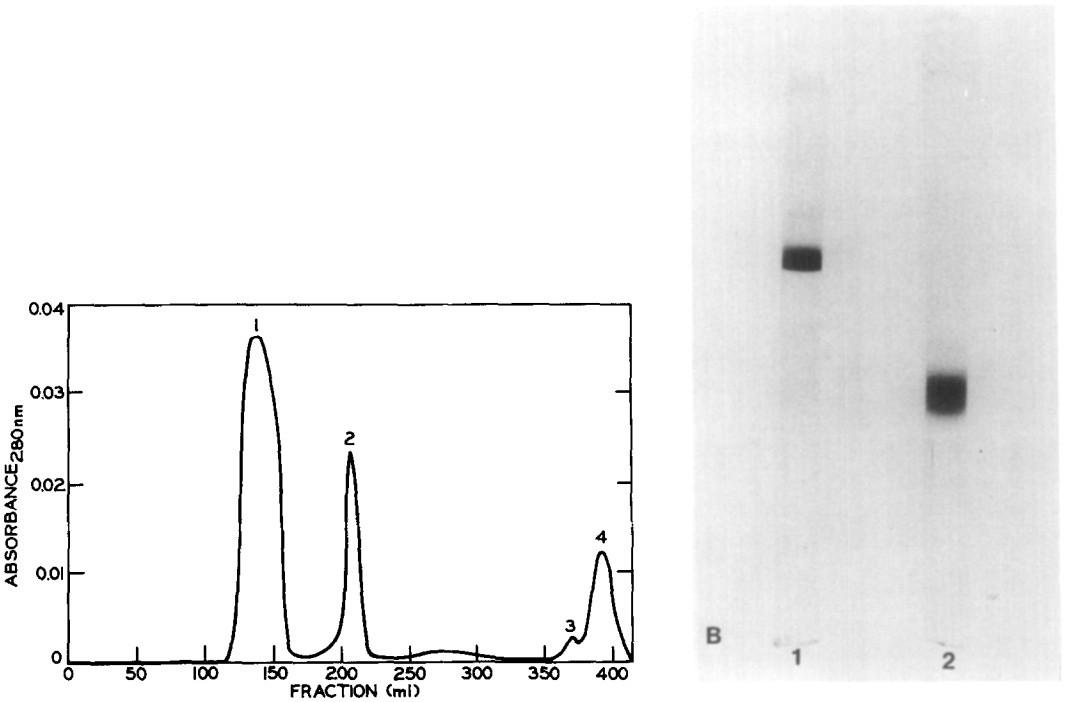

Fig. 2. Separation of the subunits by sodium dodecyl gel filtration on Bio-Gel A 1.5m. (A) the chromatogram shows the elution pattern for the subunits from the column. (B) sodium dodecyl polyacrylamide gels corresponding to elution peaks 1 and 2 from the chromatogram.

polyacrylamide gels clearly show that the glycoprotein is well separated from the catalytic subunit, and there is no cross-contamination as has been the case in most of the earlier studies. When the subunits were run by isoelectric focusing on the gels, the glycoprotein focused into nine discrete bands, accounting for all except one band seen with the holoenzyme (Fig. 3A). The one missing band is obviously the catalytic subunit.

The most likely explanation of the heterogeneity seemed to us to be variation in the oligosaccharides on individual identical polypeptide chains, since there is considerable preservation of the amino acid compositon and N–terminal amino acid (in the case of the glycoprotein) among species (14). Variation of the sialic acid content of the polypeptide chains of human fibrinogen has been demonstrated and has been offered as an explanation for the microheterogeneity of human fibrinogen seen on isoelectric focusing. Since our glycoprotein is known to contain sialic acid (13), differences in sialic acid content on individual polypeptide chains of the glycoprotein were

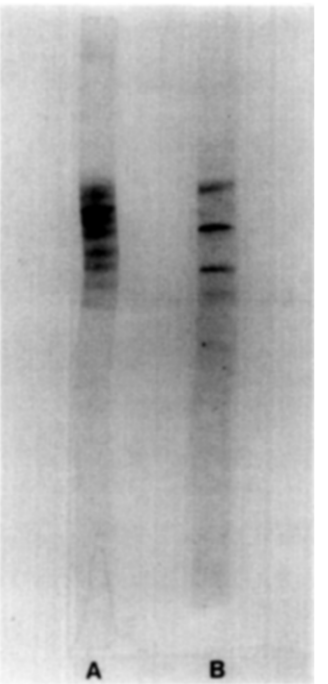

Fig. 3. The isoelectric focusing gels for the neuraminidase-treated glycoprotein. One-hundred μg of glycoprotein subunit was applied to each isoelectric focusing gel. A is the control gel, and B is the neuraminidase-treated glycoprotein.

investigated. Neuraminidase treatment of the glycoprotein removed all of the sialic acid as indicated in Table I. The resulting isoelectric focusing pattern for the neuraminidase-treated glycoprotein showed a reduction in the number of bands from nine in the control to four in the treated sample as well as a change in the focusing pattern (Fig. 3).

Apparent heterogeneity could arise artifactually from multiple equilibria or irreversible binding between the carrier ampholytes and the glycoprotein. Righetti and Drysdale (16) suggest that this type of complex formation is suspect when the focusing pattern changes with the sample load and/or ampholyte concentration. We investigated this possibility by varying the sample concentration while maintaining a constant ampholyte concentration and found no change in the banding pattern (not shown). We have also tried to minimize the introduction of artifacts by pre-electrophoresing to remove excess persulfate, by treating the protein with urea

TABLE I

Neuraminidase Treatment of Glycoprotein from Electroplax Na,K–ATPase

	Sialic Acid nmoles/mg
Control - supernatant	N.D.
pellet	137
Neuraminidase - supernatant	150
pellet	N.D.

N.D. = not detectable

The glycoprotein (2 mg/ml) was incubated for 2 hr at 37°C with and without 40 µg/ml of neuraminidase. Sialic acid was determined as described under Materials and Methods.

only in the presence of ampholytes, and by avoiding protein contact with the cathode electrolytes. The possibility of incomplete focusing was ruled out since the banding pattern or position was the same when 2900 volt-hr or 5200 volt-hr were used. Finally, the fact that the catalytic subunit showed no microheterogeneity argues against the glycoprotein heterogeneity being an artifact arising from experimental manipulation.

Microheterogeneity of glycoproteins is not a novel event, and one should be on the lookout for it since the synthesis of oligosaccharide chains of glycoproteins is not as precisely programmed as translation and therefore does not show the same high fidelity. Variations in carbohydrate groups produced by the exchange of one sugar for another on a basically similar core structure can result in heterogeneity. Many examples in the literature appear where variations in the amount of sialic acid and fucose have been shown to be the source of microheterogeneity (17–19). This also appears to be partly responsible for the microheterogeneity of the glycoprotein of the electroplax Na,K–ATPase,, since neuraminidase treatment reduced the number of bands from nine to four. Other possible sources for this microheterogeneity seen on isoelectric focusing could be either variations of the amino sugars on particular oligosaccharide chains or the number of oligosaccharide

chains attached per polypeptide chain. These possibilities along with an analysis of the carbohydrate composition in each isoelectric band are now underway.

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