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ANALYSIS OF THE NUCLEAR ENVELOPE POLYPEPTIDES BY ISOELECTRIC FOCUSING AND ELECTROPHORESIS

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Summary: The more insoluble polypeptides of the avian erythrocyte nuclear envelope have been characterized by a two-dimensional electrophoretic procedure. Most of the polypeptides occur in two classes with isoelectric points of approximately 6.4 and 5.7 respectively. The more acidic class contains two polypeptides, P71 and one which contributes to an electrophoretic band previously identified as P55. The more basic class includes P75, P68, P61 and two or more polypeptides from the P55 band. There are four to six isoelectric point variants of each polypeptide in the more basic class, and the relative stain intensities for the variants are similar for the different polypeptides. These similarities in ionic properties suggest a chemical relationship between the polypeptides. These results are discussed in relation to the in vitro conversion of P75 to polypeptides of the same molecular weight as P68, P61 and P55.

Introduction

Electrophoretic separation of polypeptides by the properties of molecular weight and isoelectric point has become a standard methodology of great resolving power. However, the nuclear envelope polypeptides are dissolved with difficulty and have not been characterized by these procedures. We have combined a sample preparation technique utilizing sodium dodecyl sulfate (1) with two-dimensional electrophoresis under denaturing conditions (2) in order to study these polypeptides.

The polypeptides of this organelle have been partially characterized in studies utilizing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Two polypeptides, P75 and P71 (identified by approximate molecular weight in thousands), predominate (3,4). P68, a less abundant polypeptide in the erythrocyte, is a major species in the same fraction from rat liver and HeLa cells (5). P68 and two other minor species, P61 and P55, are apparent cleavage products when P75 is boiled in mild acid (6). Peptide mapping studies, performed in

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sodium dodecyl sulfate solution, indicate that P75, P71 and P61 share some primary sequences (7).

A continuing difficulty in these studies, however, has been the necessity to rely on polypeptide molecular weight as the basis for both separation and identification. The present study provides a new characterization of these polypeptides.

Materials and Methods

The chicken erythrocyte nuclear protein fraction studied in this paper was prepared as follows (6). Cells were harvested from the blood of mature chickens and the nuclei released by nitrogen cavitation. The nuclei were freed of deoxyribonucleohistone by pancreatic DNAse digestion and 1.0 M NaCl extraction. The resulting envelope fraction was washed with Triton X-100 and the DNAse and NaCl treatments were repeated. The final protein pellet was dissolved in 4% sodium dodecyl sulfate, heated for 3-4 minutes in boiling water and dialyzed overnight against 10 mM sodium phosphate (pH 7.2)/0.1% sodium dodecyl sulfate/20 mM sodium azide. This material, the sample protein, was stored at -20°C and thawed prior to each use.

The two-dimensional polyacrylamide electrophoresis system of O'Farrell (2) was used for separating the chicken erythrocyte nuclear fraction for further study. The following modifications were made. Ampholines were obtained from Pharmacia instead of LKB. The cylindrical gels were not prefocused and sample was applied at the anode. The gradient slab gels did not contain glycerol. The proteins in the first dimension were focused at 500 volts, constant voltage for 3 hours. The slab gels were electrophoresed at 60 mA per slab, constant current for 2 hours or until tracking dye reached the end of the slab.

Before the protein was focused, it was precipitated from its sodium dodecyl sulfate solution (1) and then redissolved in an 8 M urea solution containing 5% NP-40, 1% βME and Pharmalytes (pH 3-10 or 5-8) in a 1:16 dilution. Briefly, the precipitation step involved combining the sample protein in a 1 to 5 volume ratio with ice cold acetone-NH_4OH (5.3:0.3). The mixture was stirred vigorously and centrifuged at 12,000 *g* for 2 minutes in a Sorvall. The supernatant was discarded and the pellet was subjected to a stream of nitrogen to eliminate traces of the acetone-NH_4OH solution. A small volume (50 µl or 100 µl) of the 8 M-urea solution mentioned above was used to redissolve the pellet. The resuspended sample protein was centrifuged in a Beckman air-driven centrifuge at a setting of 20 (approx. 70,000-80,000 *g*) for 10 minutes just previous to focusing.

Carbamylated (8) conalbumin served as an internal standard for the two-dimensional electrophoresis system of O'Farrell. The conalbumin (2 mg/ml) was boiled in a urea solution at 100°C with aliquots removed at 2 minute intervals over a range of 0-18 minutes and then recombined.

Results and Discussion

Several procedures for sample solubilization and application were tried in this study. In the most successful method, protein, dissolved in sodium dodecyl sulfate solution, was precipitated from acetone/ammonia and redissolv-

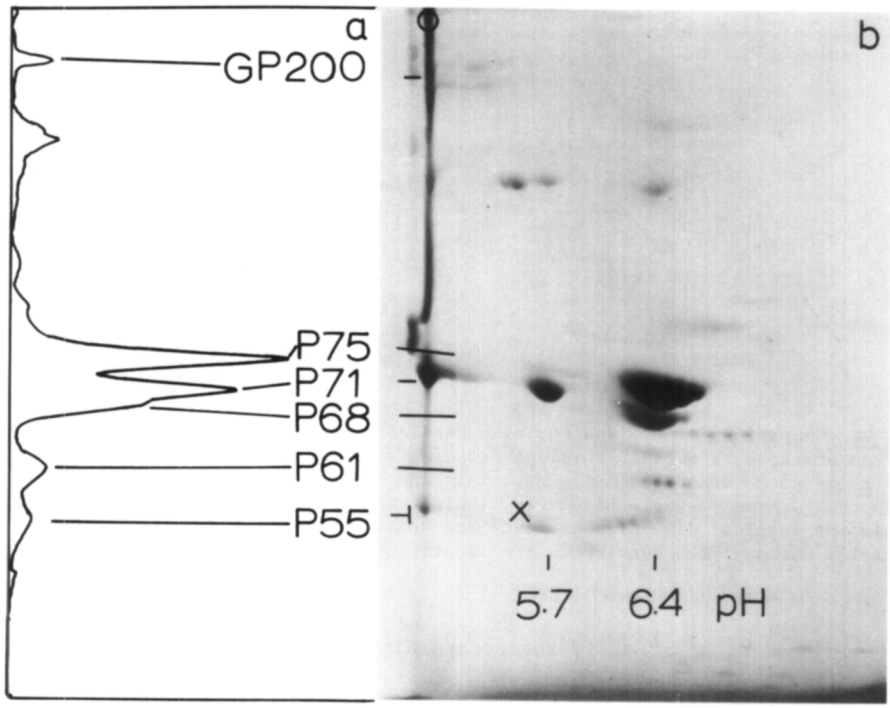

Fig. 1. Two-dimensional separation of nuclear envelope polypeptides. a Sample protein (3.6 µg) was separated by electrophoresis on a 0.5- x 8.5-cm, 5% polyacrylamide gel and the stained bands were scanned spectrophotometrically as previously described (7). The more prominent bands are identified by approximate molecular weights in thousands.

b Sample protein (160 µg prepared as described under Materials and Methods) was separated in the horizontal dimension by isoelectric focusing (pH 3-10, Pharmalytes) and in the vertical dimension by sodium dodecyl sulfate electrophoresis in a 5-20% exponential gradient gel. The more basic side of the gel which contained almost no protein is not shown. The origin is indicated by an o. The spots are identified by reference to the peaks in 1a. The pH values for the principal spots are the averages determined from three one-dimensional gels.

ed in a small volume of isoelectric-focusing buffer. This method was particularly appropriate because the sample could be prepared according to our previous studies and then quickly concentrated to the desired volume for isoelectric focusing.

In Fig. 1, the polypeptides were separated in one dimension by isoelectric focusing and in the second dimension by molecular weight. The basic region which contained only traces of protein is not shown. A typical one-dimensional sodium dodecyl sulfate electropherogram is included in Fig. 1a for identification of polypeptides. In Fig. 1b, some protein did not enter the first di-

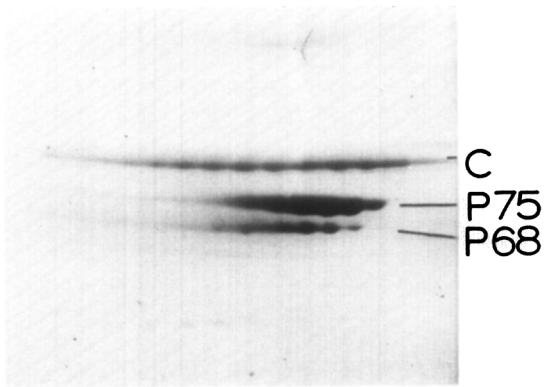

Fig. 2. Comparison of P75 and P68 with partially carbamylated conalbumin. Chicken erythrocyte nuclear envelope protein (241 µg) and conalbumin (100 µg) were prepared as described under Materials and Methods. Separation in the horizontal dimension was achieved by isoelectric focusing (pH 5-8, Pharmalytes) and in the vertical dimension as described under Fig. 1. The spots produced by partially carbamylated conalbumin are identified by c.

mension and produced a streak in the second dimension from the origin (identified by o). This material was decreased when less sample was applied. The soluble protein produced three outstanding spots corresponding to P75, P71 and P68.

P71 was the most acidic of the three and contained only one isoelectric form as evidenced by the absence of multiple spots. It was the least soluble polypeptide in the isoelectric-focusing buffer with approximately half remaining at the origin in the first dimension. The greater acidity of P71 as compared with P75 is consistent with the ratio of acidic to basic amino acids (7).

P75 and P68, in contrast to P71, dissolved almost completely. Several features were striking. P75 and P68 have similar isoelectric points and appear to contain several isoelectric point variants. In Fig. 2 the multiple forms of P75 and P68 are compared with conalbumin (pI = 6.8-7.1 (9)) which had been partially carbamylated to yield a series of isoelectric point variants differing by single charge increments (8). In both P75 and P68, the second and third spots from the basic side are the most intense. There is a marked resemblance in the distribution of spot intensities for the two polypeptides.

P68 is located in the acidic direction from P75 a distance corresponding to the loss of one net positive charge.

Although considerably smaller than P75 and P68, P61 has nearly the same isoelectric point and distribution of isoelectric point variants (Fig. 1b). P55, as identified by one dimensional electrophoresis, probably includes the polypeptide indicated by (X) and also those between (X) and P61 (Fig. 1b). The polypeptide at (X) has the same ionic and solubility properties as P71. Those polypeptides between (X) and P61, on the other hand, reflect the properties of P75, P68 and P61. Thus, most of the polypeptides of this fraction can be divided into two classes, resembling either P75 or P71.

The basis of the charge differences for the P75-like polypeptides remains to be determined. Possibilities include deamidation, acetylation, phosphorylation and glycosylation. Glycosylation is not a likely explanation because these polypeptides fail to stain either with periodic acid-Schiff stain or by a conconavalin A-horse radish peroxidase method (Shelton, K., White, C., and Higgins, L., submitted for publication). Artifactual deamidation by the acetone/ammonia precipitation step has been ruled out by experiments in which the envelope polypeptides are dissolved directly in 8 M urea/ 0.9 N acetic acid (results not shown). These polypeptides do not incorporate significant phosphate when peripheral blood is incubated with [^{32}P]phosphate in vitro (10) but they might contain phosphate groups which do not turn over in mature cells. They are major components of a urea-soluble fraction containing 2.3 µg phosphorous/mg protein (10).

The similarities of the polypeptides within these two classes are of particular interest because we have observed the in vitro conversion of P75 and P71 to polypeptides of corresponding molecular weights. Further, these conversions are specific and proceed under unexpectedly mild conditions. Upon heating at pH 3.5 for several minutes, P75 yields primarily a 61 000-dalton polypeptide but also some 68 000- and 55 000-dalton material (6). Several minutes of boiling at pH 11 converts both P75 and P71 to 55 000-dalton polypep-

tides (7). Together with the analysis of ionic properties, these results suggest that the smaller polypeptides within each class could be cleavage products of P75 and P71. Additional evidence supports the close relationship of P75, P68 and P61. P75 and P68 have very similar polypeptide interactions in the HeLa cell as revealed by polypeptide crosslinking (5). Chicken erythrocyte P75 and P68 both yield a 61 000-dalton polypeptide upon heating at pH 3.5 (6). P75 and P61 yield some common fragments upon enzymatic digestion (7). Thus, although definitive proof for a product-precursor relationship between these polypeptides has not been established the indirect evidence for this model is increasing. Such a model might be of little physiological significance, reflecting an artifact of our preparative procedures or an inconsequential in vivo breakdown. On the other hand, processing of the polypeptides might be an integral part of forming the nuclear envelope structure.

Acknowledgements

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