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N-Terminal amino acid analysis reveal peptide heterogeneity in major electrophoretic protein components of erythrocyte ghosts

HUBERTUS KNUFERMANN, SUCHARIT BHAKDI, R. SCHMIDT-ULLRICH and DONALD F. HOELZL WALLACH

Max-Planck-Institut für Immunobiologie, 78 Freiburg, Stübeweg 51 (W. Germany) and Department of Therapeutic Radiology, Division of Radiobiology, Tufts-New England Medical Center, 136 Harrison Ave., Boston, Mass. 02111 (U.S.A.)

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SUMMARY

Preparative sodium dodecylsulfate–polyacrylamide gel electrophoresis of dansylated erythrocyte membrane proteins shows that 60% of the 1-dimethylaminonaphthalene-5-sulfonyl fluorescence migrates with sodium dodecylsulfate–polyacrylamide gel electrophoresis Bands 1, 2 and 3. N-terminal amino acid analyses show that each of these bands contains at least five N-terminals. Bands 1 and 2 contain identical N-terminals. Most N-terminals appear constant, but some additional N-termini vary from one donor to the next.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis of human erythrocyte membranes reveals three major components, which together account for about 60% of the protein (peptide), according to absorptive staining procedures. These are the two "spectrin" bands, *i.e.*, Band 1 (apparent mol. wt approx 310 000) and Band 2 (apparent mol.wt approx. 290 000) and Band 3 (apparent mol. wt 93 000–87 000). Band 3 contains at least two dissimilar entities, the major erythrocyte glycoprotein and a non-glycosylated component. These can be demonstrated by selective extraction and/or differential staining.

Bands 1 and 2 have been assumed to represent single polypeptide chains but recent electrophoretic[1] and immunoelectrophoretic[2] analyses of EDTA-extracted "spectrin" suggest considerable peptide heterogeneity in these bands. However, possible proteolytic artefacts in EDTA extracts remain to be excluded; moreover such preparations contain

Abbreviation: DANSCl, 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride).

protein components other than those of Bands 1 and 2. We accordingly attack the possible heterogeneity of Bands, 1, 2 and 3 by identifying the N-terminal amino acids in these bands. For this we first dansylate membrane proteins dissolved in sodium dodecylsulfate, separate these by sodium dodecylsulfate–polyacrylamide gel electrophoresis and identify the dansylated N-terminal amino acids following acid hydrolysis.

Unless otherwise stated, we obtain all chemicals and biochemicals from Serva (Heidelberg), Boehringer (Mannheim) and Merck (Darmstadt). We obtain freshly drawn human blood (blood group O, Rh positive) in acid citrate dextrose solution. We purify erythrocytes as in refs 3 and 4, taking special care to completely remove the buffy coat of thrombocytes and leukocytes. For ghost dansylation, we modify the procedures of Gros *et al.*[5] and Gray *et al.*[6]. In this, we solubilize 15 ml human erythrocyte ghosts (approx. 5 mg protein/ml) by successive addition of 0.5 g sodium dodecylsulfate, 12.5 g urea and 7.5 ml 0.4 M phosphate buffer, pH 9.0, and follow this by heating for 2 min in boiling water. After cooling to room temperature we adjust the pH to 9.5 with 0.1 M NaOH and add 10 ml 1-dimethylaminonaphthalene-5-sulfonyl chloride (DANSCl)(Fluka, Buchs) in dimethylformamide (25 mg/ml) drop by drop with continuous pH adjustment. (Final concentrations: protein 1.8 mg/ml; sodium dodecylsulfate 1.2%; urea 5 M; sodium phosphate 75 mM; DANSCl 6 mg/ml = 26 mM = 3.3 mg DANSCl/mg protein). Dansylation proceeds for 30 min at 37 °C. Under our conditions the dansylation mixture remains entirely clear without precipitation of protein or DANSCl. After concentrating the reaction mixture to about 15 ml using a pressure filtration cell (Amicon) with a DIAFLO PM-10 membrane (Amicon), we eliminate excess DANSCl by gel filtration on Sephadex G-25 (Pharmacia, column dimension: 2.6 cm × 70 cm; elution buffer: 5 mM phosphate buffer, pH 9.0; 1% sodium dodecylsulfate, 2% sucrose). The membrane protein fraction (about 70 ml) is again concentrated to 15 ml.

Proteolysis could obviously provide false N-terminal analyses. We accordingly eliminate leukocyte contamination[3], avoid microbial growth, as tested by bacterial culture, heat inactivate samples during sodium dodecylsulfate lysis and process samples immediately. We further demonstrate lack of proteolysis during or after lysis: (a) Electrophoresis of lysed samples after storage at room temperature for up to 48 h shows no appearance of low molecular weight material or altered mobility of Bands 1, 2, 3. This is a most sensitive test for proteolysis[3], (b) Protease assays on lysates using the universal substrate Azocol (Calbiochem) are negative; *i.e.* no release of colored split products.(c) Extended heating of lysates at 100 °C and inclusion of potential protease inhibitors such as organic mercurials and ethylenediamine tetraacetate do not alter the sodium dodecylsulfate–polyacrylamide gel electrophoresis pattern.

We perform analytic sodium dodecylsulfate–polyacrylamide gel electrophoresis as in ref. 3 (gel concentration 5% total acrylamide; 2.5% cross-linking with *N,N'*-methylenebisacrylamide). Preparative sodium dodecylsulfate–polyacrylamide gel electrophoresis is essentially as in ref. 7 on 20 cm × 20 cm × 0.5 cm polyacrylamide slabs. Gels and buffers correspond to the analytical system, except that we omit the sodium dodecylsulfate in the gels and the lower buffer reservoir. We pre-electrophorese the gel slabs for 30 min at

200 mA/35 V. For preparative separations we apply 3 ml solubilized dansylated membrane protein (15 mg) per slab. We use 5 slabs (75 mg protein) and run for 7–8 h at 500 mA/80 V. After completion of electrophoresis we cut out the strongly fluorescent Bands 1, 2 and 3, homogenize the resulting gel strips and extract overnight with 100 ml 50 mM phosphate buffer, pH 8.0, 0.5% sodium dodecylsulfate per 10 ml gel with shaking. We concentrate the extracts 50-fold in an Amicon ultrafiltration cell with a PM-10 membrane and re-electrophorese aliquots analytically. We precipitate the dansylated proteins in the concentrated extracts by addition of acetone, dry and hydrolyse for 4 h and 18 h in sealed tubes, using 6 M HCl at 110 °C. We process the hydrolysates and perform two dimensional thin-layer chromatography on 5 cm × 5 cm micro polyamide plates coated on both sides (Schleicher and Schüll, Kassel) as described in refs 6 and 8, using four successive solvents: I. 1.5% formic acid in water; II. benzene–acetic acid (9:1; v/v); III. ethyl acetate–acetic acid–methanol (20:1:1; v/v/v); IV. 0.05 M Na_3PO_4 in 25% aqueous ethanol[9]. We spot hydrolysates of the individual bands on one side of the micropolyamide plates and a standard DANS–amino acid mixture on the other side. One to three thin-layer separations are usually necessary to obtain separations of identical quality on both sides of the plates. We did not investigate N-terminal tryptophan. We performed three independent series of experiments *i.e.* three different blood donors and three preparative electrophoretic separations, each followed by amino acid analysis.

We dansylate in a highly aqueous medium (25% dimethylformamide; 75% aqueous buffer). This is essential to avoid partial or completely irreversible aggregation of membrane proteins into high molecular weight complexes which cannot be dispersed by detergents and/or urea. Proteins dansylated as described migrate as brightly fluorescing bands upon sodium dodecylsulfate–polyacrylamide gel electrophoresis. These can readily be defined under ultraviolet light and can be cut precisely from the gel.

The Coomassie Blue staining pattern of preparative gel slabs after sodium dodecylsulfate–polyacrylamide gel electrophoresis of dansylated ghost proteins corresponds exactly to that obtained with analytical sodium dodecylsulfate–polyacrylamide gel electrophoresis of non-dansylated proteins[10]. Upon re-electrophoresis (Fig. 1), isolated Bands 1, 2 and 3 migrate as in the initial separation. DANSCl fluorescence and Coomassie staining yield identical distribution patterns. These data indicate lack of proteolysis.

Amino acid analysis shows at least five different dansylated N-terminal residues for all bands (Table I, Fig. 2). Most of the N-termini occur invariably, but one or two additional N-terminal amino acids vary from one donor to the next. We thus must distinguish between constant and variable N-termini the latter may reflect genetic differences between different donors, as suggested for the major human erythrocyte glycoprotein[11]. Importantly, Bands 1 and 2 have identical N-terminals (constant and variable). Band 3 with 5 constant N-termini, shared 4 N-termini with Bands 1 and 2.

Our results support those of Maddy[1] and Bøg-Hansen *et al.*[2] in demonstrating peptide heterogeneity in sodium dodecylsulfate–polyacrylamide gel electrophoresis bands. The data also point to an N-terminal homology between Bands 1 and 2. The multiple N-terminals in Band 3 further indicate considerably greater heterogeneity than hitherto

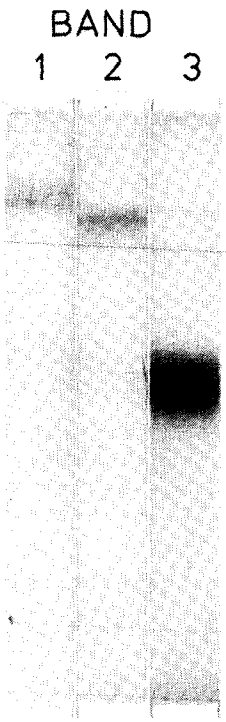

Fig.1. Re-electrophoresis of isolated, dansylated Bands 1, 2 and 3 after extraction and concentration. Coomassie blue stain. Details are given in the text.

TABLE I

CONSTANT AND VARIABLE N-TERMINAL AMINO ACIDS OF THE MAJOR ELECTROPHORETIC ERYTHROCYTE MEMBRANE COMPONENTS

	Constant	*Variable*
Band 1	Pro	Gly
	Val	Thr
	Leu	Met
	Phe	
Band 2	Pro	Gly
	Val	Met
	Leu	Thr
	Phe	
Band 3	Pro	Thr
	Val	Glu
	Leu	Gly
	Phe	Ala
	Met	

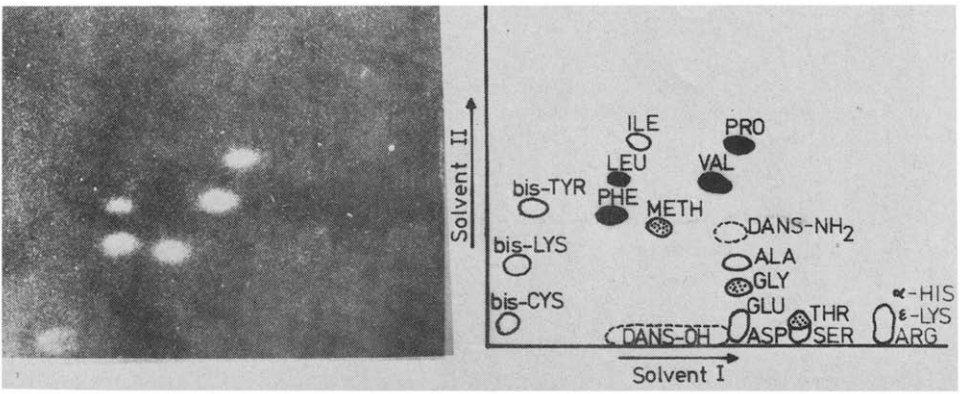

Fig. 2. Left: typical two-dimensional thin-layer chromatogram of the N-terminal DANS–amino acids of Band 1. Right: reference pattern of a standard DANS–amino acid mixture. Constant N-termini of Band 1 = solid; variable N-termini = stippled.

suspected. This heterogeneity does not involve adventitious S–S-formation, since we obtain identical sodium dodecylsulfate–polyacrylamide gel electropherograms with and without dithiothreitol.

Clearly, the bands obtained by sodium dodecylsulfate–polyacrylamide gel electrophoresis do not represent the true polypeptide composition of ghost proteins. One must consider three, possible non-exclusive explanations for our findings. First, the sodium dodecylsulfate–polyacrylamide gel electrophoresis bands may represent mixtures of unrelated peptides of similar molecular weight; second, they may represent adventitious aggregates of multiple lower molecular weight polypeptides; third, they may comprise oligomeric proteins which sodium dodecylsulfate does not dissociate into subunits. We are evaluating these possibilities by two fractionation methods ancillary to sodium dodecylsulfate–polyacrylamide gel electrophoresis, namely isoelectric focusing[12] (Bhakdi, S., Knüfermann, H. and Wallach, D.F.H., unpublished) and/or "hydrophobic" chromatography on hydroxylapatite[13] (Knüfermann, H. Bhakdy, S. and Wallach, D.F.H., unpublished).

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