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BAND 3 PROTEIN OF THE RED CELL MEMBRANE OF THE LLAMA: CROSSLINKING AND CLEAVAGE OF THE CYTOPLASMIC DOMAIN

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SUMMARY: Comparative studies were done on the cytoplasmic domain of the band 3 protein in the red cell membranes of the the human and the llama. Two approaches were used: crosslinking with o-phenanthroline/$CuSO_4$, and cleavage with 2-nitro-5-thiocyanobenzoate. o-Phenanthroline/$CuSO_4$ crosslinks the band 3 polypeptide chains in the human; in contrast band 3 in the llama is minimally crosslinked by this agent. 2-Nitro-5-thiocyanobenzoate cleaves band 3 in the human into a 23,000-dalton fragment; a similar fragment is not generated from the llama band 3. These studies show that the cysteine residue located 23,000 daltons from the N-terminus of band 3 in the human involved in these reactions is unavailable for crosslinking and cleavage in the llama. Species differences in the cytoplasmic domain of band 3 may contribute to the unusual resistance of llama red cells to osmotic, chemical and physically-induced deformation.

Human and llama red cells constitute an appropriate pair for studies on the structure and function of red cell membranes. On one hand, there are striking similarities between these two membranes in ultrastructure and biochemical makeup (1, 2) while, on the other hand, there are dissimilarities in the shape and shape behavior of their red cells (2). The llama red cells are elliptical biconvex disks (2) and are resistant to deformation by shear stress (3) and experimental conditions such as metabolic depletion and incubation in hyperosmotic media (2); these conditions are known to affect the shape of human red cells (4, 5, 6).

The llama red blood cell membrane has the same major polypeptides as the human (1, 2). There are quantitative

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differences, however, in the band 3 protein as well as in the numerical densities of the intramembrane particles of the protoplasmic fracture face of freeze fractured membranes. Both of these are two and a half times more concentrated in the llama (2).

The band 3 polypeptide of the human constitutes approximately one fourth of the total membrane protein (see 7) and has the function of anion transport (for review, see 8). Proteolysis of the human red cell membranes results in the dissection of this band into a membrane associated fragment of ~55,000-dalton and a soluble cytoplasmic fragment of ~40,000-dalton (9).

The cytoplasmic domain of band 3 bears the amino terminus of the polypeptide (10, 11), has binding sites for several glycolytic enzymes (see 12), and hemoglobin (13, 14). In the human red cell membrane the cytoplasmic domain of a fraction of band 3 polypeptides associates with band 2.1, ankyrin (15, 16). Through this binding band 3 is implicated in associations with the cytoskeleton (17, 18). As these associations constitute the submembrane structure and hence are important to red cell shape, the cytoplasmic domain of band 3 became of interest for our studies. We investigated this domain of band 3 protein in the llama in an attempt to explain the unusual rigidity of its red cell membrane. We investigated this domain with the two agents, o-phenanthroline/$CuSO_4$ and 2-nitro-5-thiocyanobenzoate, known respectively to crosslink (19, 9) and cleave (9, 11) band 3 polypeptide at the cysteine residue 23,000 daltons from the N-terminus (see 12).

MATERIALS AND METHODS

Blood was obtained from llamas (*Llama glama*) and normal human donors by venipuncture, using sodium heparin or EDTA as the anticoagulant. All procedures were performed on fresh samples of blood and unless otherwise stated at 0-5°C. Centrifugations were in Beckman Model J 2-21 centrifuge. Samples were initially washed three times in phosphate buffered saline (PBS): 150mM NaCl - 5mM

sodium phosphate buffer (pH 7.4). Due to the high protein content of llama red cells (see 2), the llama packed red cells were diluted two and a half times with PBS prior to lysis. Unsealed ghosts were prepared in 5 mM sodium phosphate buffer (pH 8.0) as described (20). Chemicals were reagent grade and were obtained from Sigma.

Treatment with o-Phenanthroline/$CuSO_4$: The crosslinking experiments was performed as described (19). One volume of ghost pellet was suspended in one volume of 5mM sodium phosphate buffer (pH 8.0) and incubated with two volumes of the same buffer containing 0 or 100 μM o-phenanthroline, 20 μM $CuSO_4$ (to make a final concentration of 50 μM o-phenanthroline, 10 μM $CuSO_4$) at 23°C for 30 minutes.

Treatment with 2-Nitro-5-thiocyanobenzoate (NTCB): Cleavage with NTCB was performed as described (9). Ghost membranes were stripped of their peripheral proteins with NaOH as described (21). One volume of packed ghosts was diluted with nine volumes of 0.1 N NaOH at 0°C and next washed twice with 5 mM sodium phosphate buffer (pH 8.0). The pellet was resuspended to 1.0 ml volume in the same buffer. A 100 μl aliquot of sample was mixed with 25 ml of 4% sodium dodecyl sulfate and 50 μl of 10 mM sodium phosphate buffer (pH 8.0) containing 0 or 4 mM NTCB and the mixture was incubated at 23°C for 15 minutes. 25 μl of 500 mM sodium borate buffer was added (pH 9.0) and the mixture was incubated overnight at 37°C (final concentrations for NTCB, 1 mM; sodium dodecyl sulfate, 1%).

Polyacrylamide Gel Electrophoresis: Reactions were terminated and samples were prepared for electrophoresis by adding one volume of "detergent concentrate" to four volumes of sample. The "detergent concentrate" contained 5% sodium dodecyl sulfate (SDS), 50% sucrose, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 50 mM pyronin Y, and 200 mM dithiothreitol as described (20). Dithiothreitol, however, was left out of the concentrate for membrane samples obtained from the crosslinking experiment with o-phenanthroline/$CuSO_4$; these membranes were incubated further for 20 minutes at 37°C. Aliquots equivalent to 40 μg of protein were loaded on gels and electrophoresis was performed according to Fairbanks et al. (20) with the following modifications. Samples obtained from the crosslinking experiment were electrophoresed on composite gels of 3.0% acrylamide plus 0.4% agarose prepared according to Peacock and Dingman (22). Samples obtained from the cleavage experiment with NTCB were electrophoresed on gels containing 5.6% acrylamide. In all these experiments N, N'-methylene-bisacrylamide concentration was 3.75% of that of the acrylamide monomer and gels contained 0.2% sodium dodecyl sulfate. The gels were stained with Coomassie blue R and scanned as described (20).

RESULTS AND DISCUSSION

Incubation with o-phenanthroline/$CuSO_4$ catalyzes the crosslinking of human spectrin (bands 1 and 2) and ankyrin (band 2.1) into higher molecular weight oligomers and band 3 into homodimer (19; see Fig. 1, top panel). In contrast, band 3 in the llama is minimally crosslinked by the same treatment, while

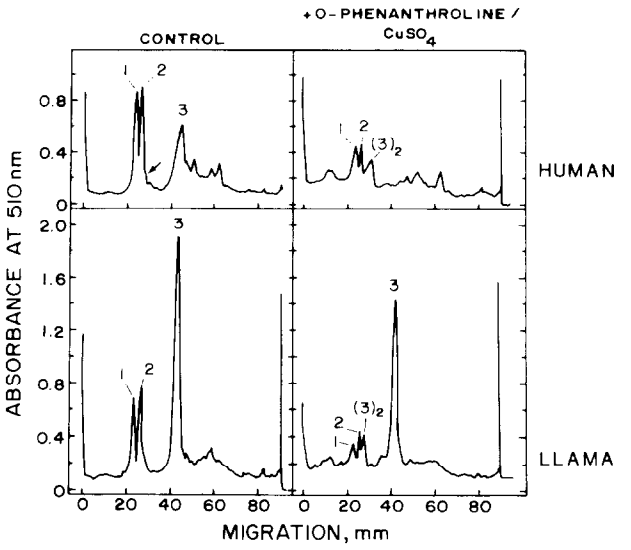

Figure 1: Crosslinking of the membrane polypeptides with o-phenanthroline/$CuSO_4$. Membranes were reacted without (left scans) and with (right scans) 50 μM o-phenanthroline, 10 μM $CuSO_4$, and solubilized in "detergent concentrate" lacking dithiothreitol and containing sodium dodecyl sulfate to final concentration of 1% as described in Materials and Methods. Aliquots (40 μg protein) were electrophoresed on 3.0% acrylamide plus 0.4% agarose composite gels containing 0.2% sodium dodecyl sulfate. Densitometric scans of the Coomassie blue stained bands on these gels show that spectrin (bands 1 and 2) and band 3 are prominent. Ankyrin (band 2.1) is at the leading edge (arrow) of band 2. Top panel, human; bottom panel, llama.

spectrin (bands 1 and 2) is crosslinked to higher molecular weight species (Fig. 1, bottom panel). 2-Nitro-5-thiocyanobenzoate (NTCB) efficiently cleaves band 3 of the human by S-cyanylation into a 23,000-dalton fragment from the N-terminus (10, 9, 11; see Fig. 2, top panel). In llama cleavage by NTCB, slightly reduces the quantity of band 3 in the membrane pellet and generates new bands of molecular weights of approximately 80,000-dalton and 74,000-dalton. However, a fragment corresponding to 23,000-dalton is not generated (Fig. 2, bottom panel).

Kobashi and Horecker (23) first demonstrated that o-phenanthroline copper complex stimulates the air oxidation of

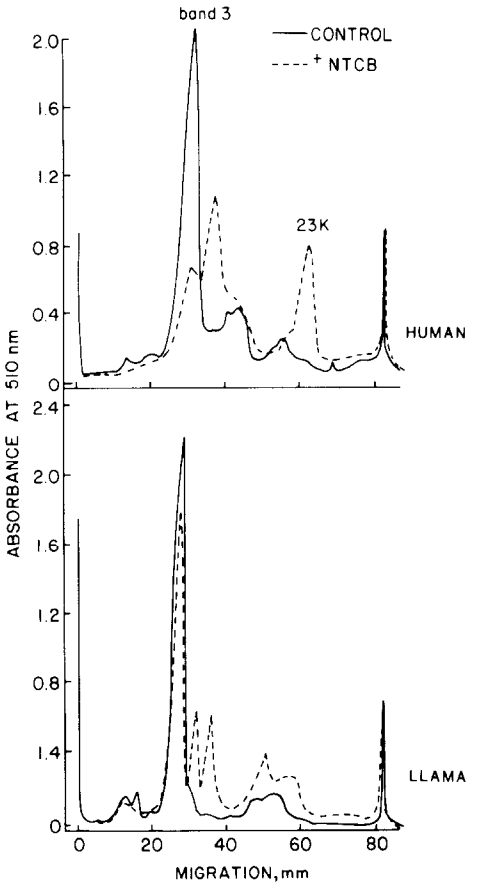

Figure 2: Cleavage of membrane polypeptides with 2-nitro-5-thiocyanobenzoate (NTCB). Ghosts were first stripped of their peripheral membrane proteins, membrane pellets were next reacted without (————) or with (-------) 1 mM NTCB, and solubilized in "detergent concentrate" containing sodium dodecyl sulfate to final concentration of 1% as described in Materials and Methods. Aliquots (40 μg protein) were electrophoresed on gels containing 5.6% acrylamide and 0.2% sodium dodecyl sulfate. The integral membrane protein, band 3, is prominent on densitometric scans of Coomassie blue stained gels of control pellets. Spectrin (bands 1 and 2) and ankyrin (band 2.1) are not represented as they have been extracted by alkaline treatment. Top panel, human; bottom panel, llama.

sulfhydryl groups of the active site of rabbit muscle aldolase creating intramolecular disulfide bonds. Human band 3 polypeptides, however, are crosslinked by this agent into dimers through the formation of intermolecular disulfide bonds (19). The

reactive sulfhydryl group of band 3 is approximately 23,000 daltons from the amino terminus. This cysteine residue is the site of disulfide crosslinking of band 3 chains by air oxidation (19). It is also particularly susceptible to peptide bond cleavage by 2-nitro-5-thiocyanobenzoate (9, 11). The fact that band 3 in the llama is neither crosslinked nor cleaved at this position (Figs. 1 and 2) suggests that this cysteine may be absent or inaccessible in the llama and hence not a critical part of band 3 structure. Band 3 is the anion transport protein of the erythrocyte membrane (see 8). A titration of anion transport sites shows a similar number in llama and human; furthermore, the anion transport capacity is similar in human and llama cells (see 2). Our data confirm that the cytoplasmic domain of band 3 is not essential to anion transport function (12, 24). Genetic variation in this domain are also common findings in normal individuals (25, 12).

The cytoplasmic domain of band 3 in the llama may have a different structure from that of the human. This difference may effect a different mode of association with the cytoskeletal proteins thereby contributing to the unusual shape stability of this red cell. The cytoplasmic pole of band 3 polypeptide in the human red cell through the binding of ankyrin, band 2.1, associates with the cytoskeletal proteins (15, 17). A different structure of this domain of band 3 in the llama could conceivably result in higher affinity binding of ankyrin, thereby providing an explanation for the finding that llama membranes incubated with EDTA retain ankyrin in the pellet fraction (2).

An alternative explanation for the rigidity of the llama red cell could be provided by the high levels of band 3 polypeptide in the llama (2). The high concentration of band 3 in this membrane could in turn affect associations with ankyrin and the

cytoskeletal proteins. Furthermore, the close packing of the integral membrane protein, band 3, could facilitate protein-protein associations within the bilayer. We have previously shown (2) that the band 3 of the llama, unlike that of the human, is not extracted by the nonionic detergent, Triton X-100.

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