Biochimica et Biophysica Acta, 602 (1980) 323—330

BBA 78970

CONTRIBUTION OF WHOLE CELL AND CYTOPLASMIC POLYPEPTIDES TO APPARENT RED CELL MEMBRANE ALTERATIONS

NANCY SAUBERMAN and L. MICHAEL SNYDER

Hematology Division, St. Vincent Hospital, 25 Winthrop St., Worcester, MA 01604 (U.S.A)

(Received March 17th, 1980)

Key words: Aging; Membrane protein; Metabolic depletion; Xerocytosis; N-Ethylmaleimide; (Red cell)

Summary

We have compared densitometric tracings of whole cell, cytoplasmic and membrane polypeptide electrophoretic patterns in an attempt to distinguish atypical partitioning from intrinsic membrane polypeptide changes occurring as a result of reticulocyte enrichment, metabolic depletion, *N*-ethylmaleimide treatment and hereditary xerocytosis. We report that membrane alterations seen in a reticulocyte-enriched population of normal cells are present in the whole cells prior to membrane isolation. Some of the membrane alterations in metabolically depleted cells and all of those in *N*-ethylmaleimide-treated cells are traced to modifications in the partitioning of polypeptides between membranes and supernatant (cytoplasm) at hemolysis.

The power of this approach in resolving the sources of apparent red cell membrane protein alterations is demonstrated in studies with hereditary xerocytes. Suggested altered partitioning of these cells described earlier (Sauberman, N., Fortier, N.L., Fairbanks, G., O'Connor, R.J. and Snyder, L.M. (1979) Biochim. Biophys. Acta 556, 292—313) is further documented and found to be unrelated to the younger cell population or slight metabolic depletion that occurs during the washing of xerocytes prior to hemolysis.

Introduction

We have previously published a study [1] which includes polyacrylamide gel electrophoresis patterns of whole red cells, hemolysate supernatants and isolated membranes from hereditary xerocytes and normocytes. After visual inspection of this slab gel we concluded that xerocytes had an abnormal whole cell pattern and that atypical partitioning at hemolysis could account for some of the alterations present in the pattern of isolated membranes. Since reticulocyte enrichment and metabolic depletion were both found to affect the normal

ghost protein pattern [1], we wondered whether such effects could be traced to altered partitioning of polypeptides at hemolysis. Also, partitioning studies of both reticulocyte-enriched and metabolically depleted xerocytes promised to further our understanding of their altered protein partitioning.

Methods

Membrane isolation in Tris buffer, electrophoresis on Maizel-Laemmli type cylindrical gels and densitometric scanning were performed according to procedures described earlier [1]. Whole hemolysates and supernatant samples were prepared for electrophoresis as follows. After 1 ml of washed, packed cells was hemolyzed in 30 ml of buffer, 1 ml aliquots of the hemolysate were removed. Following centrifugation, additional 1 ml aliquots of the supernatant were removed. To each of these samples 0.25 ml of a reducing detergent solution [1] was added and they were immediately placed in a boiling-water bath for 3 min. After cooling, additional reducing agent (40 μl of 1 M dithiothreitol) was added. These samples were then divided and frozen. All samples were boiled again immediately prior to gel application as described in Ref. 1 and 125 μl were applied to gels.

Results

Fig. 1. depicts the effect of reticulocyte enrichment on normal whole cell (a, top fraction; b, bottom fraction) and cytoplasmic (c, top; d, bottom) patterns. The corresponding ghost patterns shown elsewhere [1] have elevated staining of selective components throughout the 70 000—120 000 dalton region, especially in the band 3 shoulders and 4.1b. In the top fraction (Fig. 1a), we see a similar enrichment of polypeptides at the high molecular weight edge of band 3 and the second component below band 3 (arrow), which may be 4.1b, compared to the older, bottom fraction (Fig. 1b). The cytoplasmic

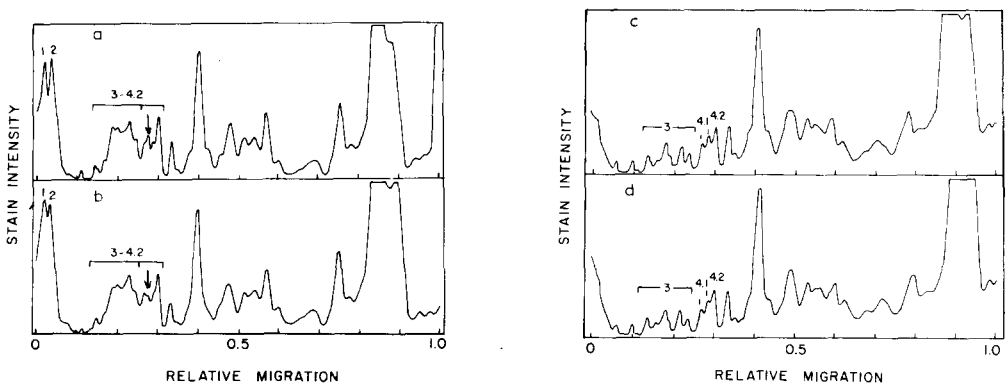

Fig. 1. Effect of reticulocyte enrichment on electrophoretic pattern of whole cells and their corresponding hemolysate supernatants. Reticulocyte enrichment was performed by the modification of Murphy's method described previously [1]. Whole cells from top (a) and bottom (b) fractions; hemolysate supernatant from top (c) and bottom (d) fractions. Arrows point to the second component below band 3 which may be band 4.1b.

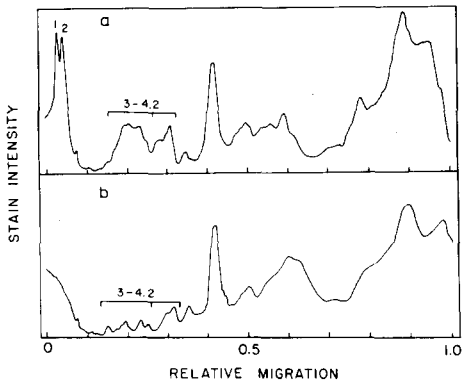

Fig. 2. Whole cell and supernatant fraction following metabolic depletion. Protocol for metabolic depletion of erythrocytes has been described elsewhere [1]. Whole cell (a) and supernatant (b).

protein patterns are essentially identical (Fig. 1c, top fraction and 1d, bottom fraction).

Studies of 24-h metabolically depleted cells were less conclusive. Even though the whole cell (Fig. 2a) and cytoplasmic (Fig. 2b) patterns were qualitatively identical to those of fresh cells, the resolution of separate polypeptides was not as clear. The slight decrease in quantity of supernatant proteins in Fig. 2b (compared, e.g., to Fig. 1c and d) is consistent with excess protein in the ghost fraction from metabolically depleted cells [1].

There has been speculation that treatment of whole cells with *N*-ethylmaleimide results in altered electrophoretic patterns of their membranes because of enhanced retention of cytoplasmic components [2,3]. In Fig. 3 we give evidence in support of this. Additional polypeptides present in the ghosts of treated cells (Fig. 3b) seem to be balanced by fewer bands in the corresponding regions of the cytoplasmic pattern (Fig. 3d). In the band 3 to 4.2 region, the whole cell patterns are unchanged by this treatment (not shown) while the

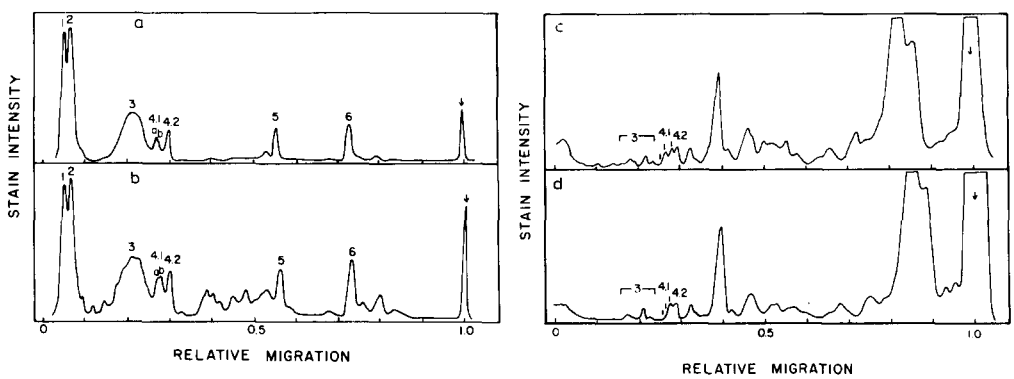

Fig. 3. Effect of incubating whole cells in *N*-ethylmaleimide. Washed erythrocytes (a 35% suspension) were incubated at 37°C for 2 h in phosphate-buffered saline which had been made 10 mM in *N*-ethylmaleimide. After two more washings in Tris-saline, the incubated cells were lysed and electrophoresed in the usual manner. Membranes from untreated (a) and treated (b) cells. Supernatant samples collected after lysis of untreated (c) and treated (d) cells.

ghosts have extra shoulders on band 3 and the 4.1a:4.1b peak-height ratio resembles that of xerocyte membranes. Besides in these, we have only seen the latter effect in pre-splenectomized hereditary spherocytes and post-therapeutic megaloblastic erythrocytes [1]. From a comparison of the cytoplasmic and membrane scans in Fig. 3, one of the polypeptides in the band 4.1 region appears to be absent from the treated cytoplasmic sample (Refer to Fig. 1c and d). Whole cells scans have three bands in this region between bands 3 and 4.2 (refer to Fig. 2a and b), while the corresponding membrane and cytoplasmic fractions show two (bands 4.1a and 4.1b). There may be reciprocal appearance of the slowest migrating of the 4.1 bands between cytoplasmic and membrane fractions. A gap between 4.1 and 4.2 in the membrane pattern not seen in that of the whole cell suggests that the fastest migrating of the three 4.1 proteins, '4.1c', is lost to the cytoplasmic fraction at hemolysis. The corresponding cytoplasmic scans seem to confirm this, since the two components here are spaced such that the slowest one appears to be missing while the other two are present.

Fig. 4. shows the band 3 to 4.2 zones from whole cells of xerocytes (Fig. 4a) and normal (Fig. 4b) erythrocytes. Eight separate polypeptides within the band 3 region and four in the 4.1 to 4.2 region are indicated. Several specific alterations in the xerocyte pattern are clearly resolved here. The scans of the cytoplasmic fractions (Fig. 4c, xerocyte; Fig. 4d, control) reflect a higher total protein concentration with a greater relative increase in the fastest migrating band 3 component. Profiles from membranes isolated from xerocytes shown elsewhere [1] have a characteristic elevation in band 3 shoulder proteins in addition to band 4.1b. From the scans of all three of these fractions, it seems that electrophoretic differences between xerocyte and control membranes are present in the whole cell and that increased loss of polypeptides at hemolysis partially normalizes the electrophoretic profile of the isolated xerocyte membranes. In an attempt to quantify this apparent trend toward normalization of the staining intensity of band 3 proteins as a result of membrane isolation, we calculated the stain-intensity ratio of spectrin to band 3 for whole cell and membrane fractions (Table I). These data confirm our previous estimate that the band 3 elevation seen in xerocyte membranes is even greater in whole cells

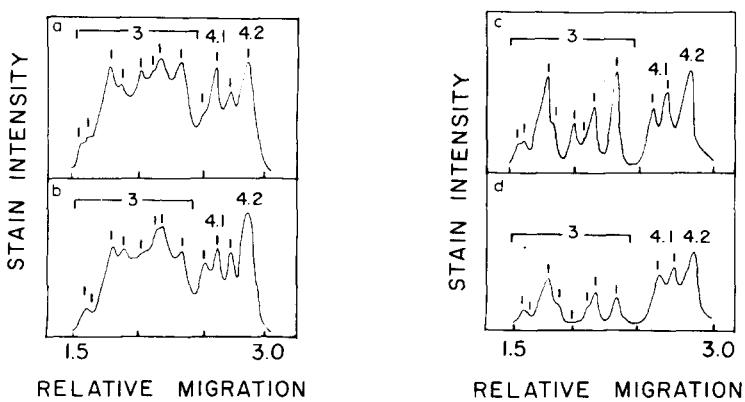

Fig. 4. Band 3 to 4.2 zone from whole cell and hemolysate supernatant electrophoretic patterns. Xerocyte and control whole cells (a,b) and supernatant (c,d).

TABLE I

SPECTRIN-TO-BAND 3 STAIN INTENSITY RATIOS OF HEMOLYSATES AND ISOLATED MEMBRANES

Four values are from duplicate gels (paired) of samples from two separate membrane isolations. The values for isolated membranes are higher than those reported previously [1], since the minor peaks on the leading edge of band 2 were included in the spectrin peak area.

	Hemolysate	Membrane
Control	1.25	1.38
	1.25	1.44
	1.20	1.33
	1.16	1.15
Xerocyte	1.05	1.30
	1.10	1.26
	1.00	1.11
	1.04	1.21

prior to membrane isolation. From the relative stain intensities of spectrin and band 3 shown in Table II, we have calculated that 82% of xerocyte band 3 is retained at hemolysis compared to 89% in the control.

Since xerocytes tend to represent a younger, slightly metabolically depleted (after 1 h in vitro) population of cells [1,4], we attempted to pinpoint specific changes due to these factors by noting the effect of further enrichment of young cells and metabolic depletion on the electrophoretic profile of xerocyte whole cell, cytoplasmic and ghost fractions. The major difference between the two xerocyte fractions is the increased stain intensity of a polypeptide at the lower, faster migrating edge of band 3 (Fig. 5a). The cytoplasmic samples showed no major difference. Metabolic depletion resulted in a blurring of the whole cell profile similar to that seen in control cells (Fig. 2) and a significant decrease in the amount of proteins lost to the supernatant at hemolysis, especially when compared to the loss which is normal for xerocytes (Fig. 4c).

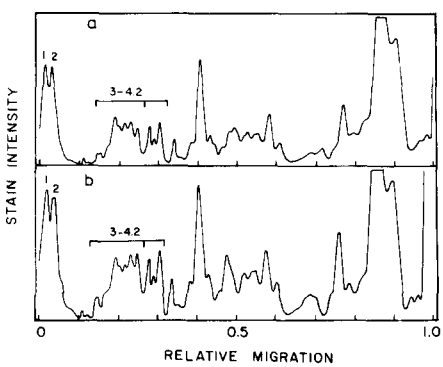

Fig. 5. Reticulocyte enrichment of xerocytes. Effect on electrophoresis pattern of top (a) and bottom (b) whole cells fractions.

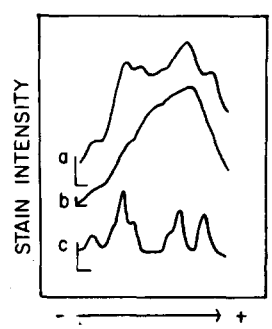

Fig. 6. Additivity of gel scans contours of hemolysate, ghost and supernatant band 3 zone. Hemolysate (a), ghost (b) and supernatant (c) fractions.

TABLE II

RELATIVE PROTEIN CONTENT OF SAMPLES BEFORE AND AFTER MEMBRANE ISOLATION

Each value is the average gel scan integration for one sample electrophoresed on two separate occasions.

Bands	Hemolysates (stain units)		Isolated membranes (stain units)		Fraction membrane bound (%)	
	Control	Xerocytes	Control	Xerocytes	Control	Xerocytes
1 + 2 *	21.6	21.9	21.8	21.5	100	98
3	17.3	20.4	15.4	16.8	89	82

* The sample loading on each gel was adjusted so that the integrated values for band 1 + 2 would be equivalent when scanned at the same gain.

TABLE III

SUMMARY OF ALTERATIONS IN ELECTROPHORESIS PROFILE OF BAND 3 TO 4 REGION

Increase (↑) or decrease (↓) or no change (—) in peak height compared to control. Band 4 refers to bands 4.1 and 4.2.

	Whole hemolysate	Supernatant	Ghost	Bands involved
Reticulocyte enrichment	↑	—	↓	3 and 4
Metabolic depletion	—	↓	↑	3 and 4
N-Ethylmaleimide treatment of red cells	—	↓	↑	3 and 4
Xerocytosis	↑	↑	↑	only 3 higher in supernatant
Reticulocyte enrichment of xerocytes *	↓	—	—	decrease in component at 3 front
Metabolic depletion of xerocytes **	—	↓	↑	3

* Compared to xerocyte bottom fraction which is reticulocyte depleted.
** Compared to fresh xerocytes.

The profile of the ghost fraction described elsewhere [1] is noticeable enhanced in band 3 shoulder proteins as predicted by the whole cell and supernatant scanning data.

Discussion

Table III is a summary of alterations in the band 3 to 4.2 zone of the electrophoresis pattern of whole cell, supernatant and ghost fractions described here. Reticulocyte enrichment of normal cells and xerocytosis were cases in which ghost pattern alterations were present in the whole cell pattern and therefore may indicate an altered membrane protein composition. Metabolic depletion and *N*-ethylmaleimide treatment of whole cells, on the other hand, seem to yield abnormal ghost patterns as a result of altered partitioning of polypeptides at hemolysis, and probably do not reflect an abnormal membrane protein composition.

A question that remains to be answered is whether the pattern alteration in

xerocytes can be explained entirely by the younger cell population. The reticulocyte-enrichment study designed to address this question gave anomalous results in that at least one protein decreased with respect to the others in the band 3 zone (Fig. 5). The creatine-approximated age of the most dense xerocytes (3.2 μmol/g hemoglobin) is considerably younger than that of the least dense control fraction (2.0) [1]. For this reason, comparison of even the most dense xerocyte fraction with the least dense control may not be valid. It could be that xerocytes are released from the marrow at an earlier stage of their development than that which occurs with normal cells. While in circulation the electrophoretic profile of xerocytes may change as a result of new proteins being synthesized [5].

Band 3 heterogeneity. The 90 000—120 000 dalton region of the ghost electrophoresis pattern has traditionally been referred to as band 3 [6—8]. The extensive area in the gel occupied by this band was once attributed to heterogeneity in glycosylation of a single protein moiety [9]. In addition to laboratory confirmation of carbohydrate differences [7,10—12], considerable evidence for the existence of more than one protein in this region has accumulated through the response of the zone's electrophoresis pattern to treatment with proteolytic enzymes [13—15], oxidizing agents [16], endogenous protein kinase [17], concanavalin A [17—21], 0.1 M NaOH [15], low ionic strength EDTA [1] and non-ionic detergent, Triton X-100 [1]. Two-dimensional electrophoresis patterns [22,23] yield many spots in this zone and at least one group [24] besides ours [1] has reported several distinct bands on one-dimensional gels.

We have previously described minor components in the 90 000—120 000 dalton region of the electrophoresis pattern of EDTA extracts from isolated membranes [1]. The suggestion of extrinsic protein contribution to this region of the membrane pattern prompted further investigation into the origin of these minor proteins. In Fig. 6 the polypeptides in this region before (a) and after (b) membrane isolation from whole cells are shown. Here we note the supernatant peak in c aligns with the pronounced shoulders of the whole cell pattern in a. The band 3 profile of the ghost pattern (b) appears to represent the difference between whole cell and cytoplasmic scans with the minor shoulders in the ghost pattern being residual cytoplasmic polypeptides retained at hemolysis. We suggest that the cytoplasmic components retained at hemolysis account for some of the heterogeneity of the band 3 zone and the equivalent zone of rabbit erythrocyte membrane patterns described by Vimr and Carter [25].

Band 4.1 heterogeneity. Recently, the protein referred to as band 4.1 has been linked to the in vitro reassociation of the spectrin-actin meshwork and hence may play a key role in red cell cytoskeleton function [26—29]. The enhanced resolution of the Maizel-Laemmli type gel system [30—31] has enabled the careful worker to resolve this band into two components, 4.1a and 4.1b [1,32]. We have observed variation in the relative peak heights of these two bands in connection with red cell aging, metabolic depletion, and various disease states [1]. In this paper, we propose that a differential retention of these two 4.1 bands at hemolysis accounts for the enhanced 4.1b peak height relative to 4.1a in the membrane fraction of *N*-ethylmaleimide-treated cells

Fig. 3). Even though the excessive retention of 4.1b at hemolysis is not obvious in any other manipulations reported here, we cannot rule out the possibility that the 4.1b in xerocyte membranes in excess of that expected for the creatine-estimated age and metabolic status is due to a similar phenomenon.

Acknowledgements

This work was supported by NIH Grant No. R01 HL19933 and NIH General Grant No. 5-S07 RR-5660. We are grateful to Drs. Grant Fairbanks and Normand L. Fortier for their criticisms and suggestions, to Jane Griesback for photographic assistance and Ethel Johnson for typing the manuscript.

References

1 Sauberman, N., Fortier, N.L., Fairbanks, G.F., O'Connor, R.J. and Snyder, L.M. (1979) Biochim. Biophys. Acta 556, 292—313
2 Allen, D.W. and Cadman, S. (1976) Proc. Soc. Exp. Biol. Med. 152, 318—321
3 Liu, S.C., Fairbanks, G. and Palek, J. (1977) Biochemistry 16, 4066—4074
4 Fairbanks, G., Dino, J.E., Fortier, N.L. and Snyder, L.M. (1978) in Erythrocyte Membranes: Recent Clinical and Experimental Advances (Kruckeberg, W.C., Eaton, J.W. and Brewer, G. J. eds.), pp. 173—184, Allan, R. Liss, Inc., New York
5 Light, N.D. and Tanner, M.J.A. (1978) Biochim. Biophys. Acta 508, 571—576
6 Furthmayer, H., Kahane, I. and Marchesi, V.T. (1976) J. Membrane Biol. 26, 173—187
7 Jenkins, R.E. and Tanner, M.J.A. (1977) Biochem. J. 161, 139—147
8 Steck, T.L. (1978) J. Supramol. Struct. 8, 311—324
9 Yu, J. and Steck, T.L. (1975) J. Biol. Chem. 250, 9170—9175
10 Gahmberg, C.G. (1976) J. Biol. Chem. 251, 6108—6116
11 Tanner, M.J.A., Jenkins, R.E., Anstee, D.J. and Clamp, J.R. (1976) Biochem. J. 155, 701—703
12 Fukuda, M., Fukuda, M.N. and Sen-itiroh, H. (1979) J. Biol. Chem. 254, 3700—3703
13 Reichstein, E. and Blostein, R. (1975) J. Biol. Chem. 250, 6256—6263
14 Gahmberg, C.G. (1976) J. Biol. Chem. 251, 510—515
15 Jennings, M.L. and Passow, H. (1979) Biochim. Biophys. Acta 554, 498—519
16 Kahlenberg, A.(1976) Anal. Biochem. 74, 337—342
17 Roses, A.D. (1976) J. Supramol. Struct. 4, 481—486
18 Findlay, J.B.C. (1974) J. Biol. Chem. 249, 4398—4403
19 Bachi, T. and Schnebli, H.P. (1975) Exp. Cell Res. 91, 285—295
20 Tanner, M.J.A. and Anstee, D.J. (1975) Biochem. J. 153, 265—270
21 Barrat, D.G., Sharom, F.J., Thede, A.E. and Grant, C.W.M. (1977) Biochim. Biophys. Acta 465, 191—197
22 Conrad, M.J. and Penniston, J.T. (1976) J. Biol. Chem. 251, 253—255
23 Bhakdi, S., Bjerrum, O.J. and Knufermann, H. (1976) Biochim. Biophys. Acta 446, 419—431
24 White, M.D. and Ralston, G.B. (1976) Biochim. Biophys. Acta 436, 567—576
25 Vimr, E.R. and Carter, J.R. (1976) Biochem. Biophys. Res. Commun. 73, 779—784
26 Fowler, V. and Taylor, D.L. (1978) J. Cell Biol. 79, 222a
27 Tyler, J.M., Hargeaves, W.R. and Branton, D. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5192—5196
28 Ungewickell, E., Bennett, P.M., Calvert, R., Ohanian, V. and Gratzer, W.B. (1979) Nature 280, 811—814
29 Lux, S.E. (1979) Nature 281, 426—430
30 Laemmli, U.K. (1970) Nature 227, 680—685
31 Maizel, J.V. (1971) in Methods of Virology (Masamorosch, K. and Koprowski, H., eds.), Vol. 5, pp. 179—246, Academic Press, New York
32 Mueller, T.J. and Morrison, M. (1977) J. Biol. Chem. 252, 6573—6576