A Study of Membrane Protein Defects and $\alpha$ Hemoglobin Chains of Red Blood Cells in Human $\beta$ Thalassemia*

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The soluble pool of $\alpha$ hemoglobin chains present in blood or bone marrow cells was measured with a new affinity method using a specific probe, $\beta^a$ hemoglobin chain labeled with [3H]N-ethylmaleimide. This pool of soluble $\alpha$ chains was $0.087 \pm 0.017\%$ of hemoglobin in blood of normal adult, $0.11 \pm 0.03\%$ in heterozygous $\beta$ thalassemia and ranged from $0.26$ to $1.30\%$ in homozygous $\beta$ thalassemia intermedia. This elevated pool of soluble $\alpha$ chains observed in human $\beta$ thalassemia intermedia decreased 33-fold from a value of $10\%$ of total hemoglobin in bone marrow cells to $0.3\%$ in the most dense red blood cells. The amount of insoluble $\alpha$ chains was measured by using the polyacrylamide gel electrophoresis in urea and Triton X-100. In $\beta$ thalassemia intermedia the amount of insoluble $\alpha$ chains was correlated with the decreased spectrin content of red cell membrane and was associated with a decrease in ankyrin and with other abnormalities of the electrophoretic pattern of membrane proteins. The loss and presence of free thiol groups.

The soluble $\alpha$ chain pool present in all cells, from bone marrow cells to dense blood cells, was assessed by using a specific probe $\beta^a$ hemoglobin chain labeled with [3H]NEM which combines with the soluble $\alpha$ chains present in cell lysates to form $^3$H Hb, separated and evaluated by electrophoresis.

The insoluble $\alpha$ chains, remaining in the red cell ghosts after cell lysis and extensive washing of the ghosts, were evaluated by using polyacrylamide gel electrophoresis in the presence of urea and Triton X-100 (UT-PAGE) in order to separate simultaneously the various globin chains $\alpha$, $\beta$, $\gamma$, and $\delta$ and the membrane proteins (6). The standard SDS-PAGE method was also used (7).

The thiol groups of the various membrane proteins were characterized by their assay with dithiobisnitrobenzoic acid and by the binding of [3H]N-ethylmaleimide to the red cell ghost prior to UT-PAGE and autoradiography.

EXPERIMENTAL PROCEDURES

Material—Parahydroxymercuribenzoate and phenylmethylsulfonyl fluoride were purchased from Sigma, soluene was from Packard, NEM was from Behring Diagnostics, [3H]NEM was from Du Pont-New England Nuclear, and antibodies were from Cappel and Jackson.

Patients—We investigated with their informed consent seven normal adult controls, two controls with high reticulocyte counts, one splenectomized control, 19 subjects with asymptomatic heterozygous $\beta$ thalassemia, and 10 patients with $\beta$ thalassemia intermedia who were not transfused during the 3 months preceding the study. Fractionation of RBCs by density on a discontinuous Stratan gradient (8) was performed for a splenectomized patient with $\beta$ thalassemia intermedia. Bone marrow cells were obtained from two normal bone marrow donors and one patient with $\beta$ thalassemia intermedia.

Preparation of Hemoglobin Subunits—Hemoglobin A was purified by DEAE-cellulose chromatography performed in 0.2 M glycine buffer, pH 7.8 (9). The subunits of Hb A were prepared by dissociation of Hb A in the presence of parahydroxymercuribenzoate followed by ion exchange chromatography, according to the method of Bucci et al. (10, 11).

Preparation of Radioactive [3H]N-ethylmaleimide—The $\beta^a$ hemoglobin chain was reacted with [3H]N-ethylmaleimide as follows: 46.5 mg of $\beta^a$ chain in 0.2 M glycine buffer, pH 7.8, were incubated with 465 $\mu$Ci of [3H]NEM (49 Ci/mmol) during 1 h at 0°C. Nonradioactive NEM in stoichiometric concentration to the SH groups of the $\beta$ chain was then added, and the incubation was continued for 1 h to alkylate all thiol groups of the $\beta$ chains. The radioactive $\beta^a_{\text{NEM}}$ was stripped by gel filtration performed in 0.1 M phosphate buffer and stored as droplets in liquid nitrogen until used. In this condition the specific radioactivity was $9 \times 10^6$ cpm/mg of $\beta^a_{\text{NEM}}$ chains, but it could be much greater if needed.

The [3H]N-ethylmaleimide probe was able to combine to soluble $\alpha$ chains in spite of the binding of two NEM molecules at the $\beta$-93 and $\beta$-112.

1 The abbreviations used are: NEM, N-ethylmaleimide; UT-PAGE, Triton X-100-polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RBCs, red blood cells.
positions, as indicated by the SH group assay of βEM chains (result not shown). The [3H]βEM chain was homogeneous with regard to electrophoresis and α chain binding.

Measurement of the Free α Chain Pool—Blood samples anticoagulated with heparin were washed immediately with sodium chloride 0.15 M by three centrifugations performed at 4 °C; the buffy coat was not removed. The packed cells were mixed with 3 volumes of water and centrifuged at 2000 rpm for 20 min at 4 °C. The membrane-free lysate of the thawed sample was obtained by centrifugation at 100,000 × g for 30 min at 4 °C. The hemoglobin concentration was adjusted to obtain 50 mg/ml in the final incubation mixture which was composed of 0.1 M sodium phosphate, pH 7.8, and also contained [3H]pNEM chains in a given Hb/β chain molar ratio. The mixture was incubated at 0 °C usually for 1 h. A known volume of the incubation mixture, 3 μl, was subjected to cellulose acetate electrophoresis (11) performed with 0.03 M sodium phosphate buffer, pH 6.5, made 2 mM in KCN. Electrophoresis was performed for 90 min at 100 V and at room temperature. Subsequently, the strip was stained with Amido Black, destained, and cut transversely in 1-mm sections. Each fraction was incubated in a scintillating vial in the presence of 0.05 ml of water and 0.5 ml of soluhene 350 and was counted in a toluene-based scintillating fluid. The quenching for staining was corrected by using an external standard. The amount of radioactive Hb in the Hb fraction corresponded to the βEM chain recombined to the α chain pool. The proportion of free and soluble α chains present in the lysate was calculated by reference to the concentration of soluble Hb present in the sample to be analyzed:

% α chain = \frac{cpm Hb \, A^{EM}}{SA \times q \, Hb} \times 100

SA being the specific radioactivity of [3H]pNEM (cpm/μmol) and q Hb being the quantity in μmol of Hb loaded on the strip.

Preparation of Ghosts—Ghosts were prepared according to the method of Dodge et al. (12) in the presence of phenylmethylsulfonylfluoride (0.1 mM).

Gel Electrophoresis—SDS-PAGE of ghosts was performed according to Laemml (7) using a 10-15% polyacrylamide gradient. Modificaiton of the UT-PAGE described by Rovner et al. (13) was used as published previously (6).

Assay and Labeling of Membrane Protein Thiol Groups—The thiol group of red cell ghosts were assayed by using dithiobisnitrobenzoic acid (14). To characterize the various thiol groups of proteins, ghost samples containing 100 μg of proteins in 25 μl were incubated with [3H]NEM for 10 min at 0 °C in three conditions. The highly reactive thiol groups were assessed with [3H]NEM 6 × 10^-7 M. The low reactive thiol groups were estimated by preincubating the ghosts with subsaturating concentration of unlabeled NEM (1.8 × 10^-7 M) and then adding the [3H]NEM 6 × 10^-7 M. The total thiol groups were characterized by saturating amount of NEM (mixture of [3H]NEM (6 × 10^-7 M) and unlabeled NEM (1.8 × 10^-7 M). The samples were subsequently submitted to SDS and UT-PAGE, then to autoradiography, and densitometry of the film (15).

Immunodetection of Globin Chains—Blot transfer of proteins from gel to nitrocellulose was performed according to Waterbor and Harrington (16) and immunostained with the alkaline phosphatase light (1.9%) to dense cells (20.1%) was related to cell selection.

Bone marrow cells obtained from the same patient with β thalassemia intermedia, the membrane protein profile obtained upon SDS-PAGE, used as reference for comparison to UT-PAGE, showed a 23% decrease in spectrin compared with the control value, a slight decrease of band 3, a 23% increase of bands in the 4.2-5 area, and the appearance of several minor bands between band 6 and globin. The ratio spectrin/band 3 was decreased by 10% (Table I).

The electrophoretic pattern obtained by UT-PAGE is shown in Fig. 4. The proportion of various membrane proteins and of globin chains are shown in Table II and III, respectively. The proportions of various globin chains evaluated as the percentage of membrane proteins are shown in Table III. The polypeptide profile of red cell ghost proteins was identical for controls with or without splenectomy. In heterozygous β thalassemia there was only an increase in the α and β globin chains in equivalent amount suggesting the increased binding of hemoglobin A to membrane. In splenectomized patients with β thalassemia intermedia, the membrane protein evaluation showed a decrease in the proportion of spectrin (26%) and of ankyrin (34%) and an apparent increase in the actin fraction (16%), but not changed in SDS-PAGE, a high proportion of globin chains, mainly α chains and the presence of additional bands called U1-U7, located between the band 3 region and globins chains. A correlation was observed between the decrease of spectrin and the proportion of insoluble α chains associated with cell ghosts (n = 5, R = 0.89, p < 0.005).

Fig. 4 and Table IV show an example of the UT-PAGE pattern of ghost proteins obtained from red cells of a patient with β thalassemia intermedia, fractionated by a Stractan
**Fig. 1.** Electrophoresis profile of radioactivity of samples incubated with $[^3H]BH_{\text{NEM}}$. A, purified HbA. B, hemolysate of a normal individual. C, hemolysate of a patient with $\beta$ thalassemia intermedia ($O$ = origin).

**Fig. 2.** The $\alpha$ chain pool in erythrocytes of normal controls (A) heterozygous $\beta$ thalassemia (B), homozygous $\beta$ thalassemia (C) ($R$, high reticulocyte counts; $S$, splenectomy).

**Fig. 3.** Levels of $\alpha$ chain pool and of Hb F in red blood cells from a patient with $\beta$ thalassemia intermedia fractionated by density. Fraction 1 is of the light cell fraction.

**Fig. 4.** UT-PAGE stained with Coomassie Blue of RBC ghost proteins of a splenectomized patient with a $\beta$ thalassemia homozygote. RBC were separated on Stractan density gradient in 4 fractions. 1, normal with cord blood hemoglobin added; 2, fraction 1 (the lightest); 3, fraction 2; 4, fraction 3; 5, fraction 4 (the most dense); 6, total ghosts.

These results indicate that spectrin chains are not only in a lower amount but also modified in the very light and most abnormal thalassemic cells. Ankyrin may be more decreased in the lightest cells than in dense cells. In addition, some uncharacterized fractions are present in greater proportion in the light fraction of cells such as U1 or U2 which are not present in controls or in heterozygous $\beta$ thalassemia. However U3 and U4, are more increased in dense cells than in light cells. Some of the proteins such as band 3, 4.1, 4.2, and 4.9 remain in stable proportion with increasing cell density. Globin chain analysis of cell ghosts showed that $\beta + \gamma$ globin chains and $\alpha$ globin chains varied in opposite fashion. $\alpha$ chains decreased with cell density while non-$\alpha$ hemoglobin chains comprised 5.5% of membrane proteins in light cells but 10.8%
TABLE II
Proportion of membrane proteins analyzed by UT-PAGE expressed as the percentage of total membrane proteins (without globin)

| No. | Spx, splenectomized | No Spx, no splenectomized | No. of patients | U1, 2, 3, etc., uncharacterized fractions |
|-----|---------------------|---------------------------|-----------------|------------------------------------------|
| Controls | 9 | 28.6 ± 2.8 | 8.1 ± 1.5 | 350.6 ± 4.1 | 50 ± 0.5 | 0.3 ± 1.2 | 5.3 ± 0.6 | 0.4 ± 0.6 | 0.1 ± 0.3 |
| Controls spx | 2 | 28.9 ± 1.3 | 11.4 ± 0.5 | 50.6 ± 1.5 | 6.4 ± 0.3 | 0.5 ± 0.3 | 5.2 ± 3.3 | 0.5 ± 0.5 | 0.1 ± 0.1 |
| Thalassemia heterozygote no spx | 4 | 25.7 ± 3.5 | 7.5 ± 1.4 | 521.5 ± 5.8 | 0.6 ± 1.9 | 0.5 ± 1.9 | 6.4 ± 4.9 | 0.2 ± 2.2 | 1.7 ± 1.5 |
| Thalassemia intermedia spx | 8 | 19.9 ± 2 | 5.3 ± 1.5 | 492.4 ± 3.9 | 3.6 ± 0.9 | 7.1 ± 0.9 | 5.0 ± 1.4 | 0.1 ± 0.1 | 0.3 ± 0.3 |

TABLE III
Proportion of globin chains analyzed by UT-PAGE as the percentage of total membrane proteins (abbreviations as in Table II)

| No. | αγ | αγ | α | α/β |
|-----|----|----|---|-----|
| Controls | 9 | 0 | 0 | 0 |
| Controls spx | 2 | 0 | 0 | 0 |
| Thalassemia heterozygote No Spx | 4 | 0 | 0 | 0 |
| Thalassemia intermedia Spx | 8 | 1.3 ± 0.6 | 1.9 ± 0.7 | 1.3 ± 1.1 |

TABLE IV
Proportion of membrane proteins analyzed by UT-PAGE expressed as the percentage of total membrane proteins (without globin) of RBC separated on Stractan density gradient in 4 fractions from a splenectomized patient with a β thalassemia intermedia (fraction 1 is the lightest)

| Fractions | Stractan | Spectrin | Ankyrin | 4.2 + 3 + 4.1 | U1 | U2 | U3 | U4 | Actin | U6 | 4.9 | U7 |
|-----------|----------|----------|---------|-------------|----|----|----|----|-------|----|-----|----|
| 1         | 12       | 3        | 44.1    | 5.2         | 6  | 9  | 9.5| 6.6| 3.3   | 1.3| 1.8 |
| 2         | 17.4     | 5.2      | 46.2    | 3.2         | 3.5| 6.6| 6.5| 6.5| 2.1   | 1.2| 0.7 |
| 3         | 19.8     | 4        | 43.5    | 1.8         | 3.5| 4.6| 5.2| 11.2| 1.7   | 2.1| 1.3 |
| 4         | 18.6     | 5.5      | 43.4    | 2.6         | 3.6| 4.4| 4.9| 10.6| 2.4   | 2  | 2  |
| Total     | 15.9     | 5.2      | 46.3    | 3.5         | 4.2| 7.3| 6.4| 6.6| 1.5   | 1.1| 0  |

TABLE V
Proportion of globin chains analyzed by UT-PAGE expressed as the percentage of total membrane proteins of RBC separated on Stractan density gradient in 4 fractions from a splenectomized patient with a β thalassemia intermedia (fraction 1 is the lightest)

| Fractions | Stractan | α + β | αγ | α | α/β | Free α chains (% α/β non-α) |
|-----------|----------|-------|----|---|-----|-----------------------------|
| 1         | 2.7      | 28.8  | 42.2| 8 | 38.7 |
| 2         | 1.7      | 38.8  | 21.8| 4.8| 16.3 |
| 3         | 4.2      | 5.3   | 21.5| 23 | 12   |
| 4         | 4.7      | 6.1   | 24  | 2.2| 13.2 |
| Total     | 2.7      | 4.1   | 30  | 4.4| 20.5 |

FIG. 5. Immunoblotting of an UT-PAGE of β thalassemic (1) and normal (2) ghosts; Coomassie Blue staining. Arrows indicate the presence of globin in β thalassemia. In normal cells globin was present mainly in spectrin and band 3.

in dense cells (Table V). The immunoblotting of UT-PAGE showed the presence of globin in various ghost protein fractions (Fig. 5) in β thalassemia intermedia.

Free Sulfhydryl Groups—The present study showed a 27% decrease of the reactive thiol groups of ghost proteins, from 74.7 nM/mg of proteins in normal ghosts to 54.3 nM/mg in β thalassemia intermedia. In the conditions used, the binding of 3H-NEM was specific to thiol groups because it was completely prevented by preincubation of ghosts with iodoacetamide (5 mM), a highly specific thiol reagent. To discriminate the very reactive from the low reactive thiol groups, we used different amounts of 3H-NEM added before, after, or with a subsaturating amount of non-radioactive NEM. The autoradiography showed, in Fig. 6 the dramatic decrease of thiol groups of membrane proteins which predominated in spectrin, ankyrin, and actin but also in uncharacterized fractions numbered 1, 2, 5, and 6 (Table VI). An abnormal band, number 3, appeared in β thalassemic cells in association with other minor bands and the partial labeling of globin, mainly α chains. Fig. 7 shows the percentages of thiol groups with high or low reactivity in spectrin and ankyrin of a normal control and of the patient who had a very high soluble α chain pool (1.3%) and a very high proportion of insoluble α chains.
Radiography of an UT-PAGE of RBC ghosts incubated with 0.1-3. ukyrin exhibited 12.2% of the low reactive thiol groups in normal cells and 5.0% in thalassemic cells. Ankyrin groups affected in addition spectrin had more low reactive thiol groups in normal decreasing from 25.2% in normal membranes to 3.5% and of low reactive thiol groups. (30%) present in red cell ghosts. The most reactive thiol groups affected in β thalassemic ghosts were that of ankyrin decreasing from 25.2% in normal membranes to 3.5% and of spectrin 6.8% instead of 13.7% in normal membranes. In addition spectrin had more low reactive thiol groups in normal cells (49.4%) than in thalassemic cells (17.5%) while ankyrin exhibited 12.2% of the low reactive thiol groups in normal cells and 5.0% in β thalassemic cells.

DISCUSSION

Soluble α Chain Pool—The amount of soluble α chains present in normal or β thalassemic cells has not been measured previously. However the incorporation of radioactive amino acids during protein synthesis in reticulocytes and subsequent separation of soluble α chains from tetrameric hemoglobins (18–24) has indirectly shown that the α chain pool was increased in β thalassemic reticulocytes. This method using protein synthesis is poorly suitable in studying the α chain present in bone marrow cells because of the cell heterogeneity and of non-heme proteins synthesized in addition to globin chains. This method is not suitable for studying the soluble α chain pool present in red cells which have lost the capability of protein synthesis. For these reasons we devised an affinity method to evaluate the soluble α chain pool present in all types of erythroid cells. The soluble α chain pool evaluated by this affinity method was relevant to the amount of free and soluble α chains present in intact cells in vivo at the time of sampling because the method fulfilled the conditions required for the quantitative evaluation of soluble α chains. The method is specific, very sensitive, and suitable for evaluating α chains in the presence of proteins other than hemoglobin. The sample processing is fast, performed at 0°C, and is not associated with a loss of soluble α chains during 3 h of blood storage or upon freezing in liquid nitrogen and thawing in the conditions used.

In normal individuals, free α chains are present in red blood cells but in very small amounts, 0.067% ± 0.017 of Hb. In the β thalassemia trait, the α chain pool was only slightly increased in spite of an unequal globin chain synthesis (α/β ratio = 2) (19, 21, 22), suggesting a very efficient removal of soluble α chains in heterozygous β thalassemia, more by proteolysis than by precipitation because of the absence of significant amounts of insoluble α chains associated with ghosts in heterozygous β thalassemia. However, selective removal of abnormal cells containing precipitated α chains could also explain the disappearance of unpaired α chains during maturation of reticulocytes and red cell aging in heterozygous β thalassemia.

In β thalassemia major, traces of soluble α chains able to combine with β chains and to form Hb A have been detected (25–27). The pool of soluble α chains observed in the present study (0.26–1.30%) was highly different from one patient to another. The amount of soluble α chains was modest with respect to total hemoglobin and to the imbalance of globin chain synthesis because of the instability of α chains (24, 27) proteolysis (23, 24, 28–30), and removal of damaged cells by the reticuloendothelial system. In this regard four splenectomized patients with a relatively severe β thalassemia as determined by blood hemoglobin level had a high α chain pool (0.80–1.30). Two splenectomized patients with the lowest α chain pool (0.38–0.48) had a very mild form of β thalassemia suggesting that the soluble α chain pool could be related to the clinical severity of the disease in splenectomized and untransfused patients.

In β thalassemia intermedia, the soluble α chains are not restricted to reticulocytes as suggested by the distribution of the non-radioactive α chain pool ranging from 2.4% in most light cells containing 32% of reticulocytes to 0.3 in dense cells containing virtually no reticulocyte (<0.5%).

In bone marrow cells, a very high proportion of α chains exists in the soluble form (10% of Hb, i.e. 20% of α chains present in Hb). This high α chain pool in the bone marrow cells of a patient with β thalassemia intermedia contrasted with the small α chain pool value in normal bone marrow cells which, however, is 2–3 folds of that observed in normal

![Graph](image_url)
blood, which is most probably related to the removal of the free and soluble α chains during maturation of normal red blood cell precursors. The much lower soluble α chain pool in reticulocytes (2.4%) than in bone marrow cells (10%) of the patient with β thalassemia intermedia is related to the precipitation and proteolysis of α chains and differential cell death during erythroblast maturation.

**Insoluble Hemoglobin Chains**—Previous studies (3, 32, 33) of red cell ghosts from β thalassemic patients revealed an increase in their globin content. Results obtained in the present study using UT-PAGE showed a peculiar globin chain pattern in β thalassemia syndromes. In heterozygous β thalassemia, the amounts of β and α globin chains present in ghosts were increased in similar proportion, suggesting that insoluble hemoglobin is increased and that most of the free α chains resulting from the unequal globin chain synthesis are removed or degraded. In this condition, the globin content of ghosts was not a simple contamination of soluble Hb because extensive washing of ghosts did not remove further hemoglobin, and ghosts from normal cells contained barely detectable hemoglobin but may more probably reflect an abnormal binding of Hb to membrane (34).

In homozygous β thalassemia, insoluble α hemoglobin chains have been found to be present in cell ghosts (3, 33). In the present study ultrasonification of cell ghosts and subsequent centrifugation did not lead to separation of globin or hemoglobin from membrane, suggesting that insoluble hemoglobin chains were bound to membrane and not present as free inclusion bodies trapped in cell ghosts after hemolysis (35). In addition we showed that non-α globin chains (β or β + γ) were also present in ghosts and that their amount increased with cell density from 5.5% of total membrane proteins in light cells to 10.8% in dense cells. In contrast, the insoluble α chains decreased from 44.2 to 24% with cell density and the α/α+α ratio of globin present in ghosts dropped from 8 to 2.2. The increase in β and γ chains in membrane ghosts in cells with increasing density indicates a reduced solubility of hemoglobin tetramers which may be related to increased oxidation in the thalassemic cells.

**Membrane Proteins**—In β thalassemia intermedia changes in membrane proteins were amplified in the lightest cells which had a low hemoglobin content and a high amount of insoluble α chains. Both SDS-PAGE and UT-PAGE showed a reduction of spectrin and an increase of uncharacterized fractions. Band 3 was also reduced by SDS-PAGE but to a lower extent than spectrin chains. Consequently, the ratio Spectrin/band 3 decreased slightly in β thalassemia as shown in Table I. A slight deficit of spectrin has been also shown by Shinar et al. (33). Part of spectrin binding to intrinsic membrane proteins is reduced as shown by Platt and Falcone (36) for cells containing unstable hemoglobin, due to an increased oxidation. It is well known that oxidation of spectrin will increase the proportion of spectrin high molecular weight complexes (37, 38) and impair the assembly of cytoskeleton (39, 40). Diamide at very low concentration (3 μM) prevents the formation of the spectrin-4.1 complex in the presence of actin. Erythrocytes with inclusion bodies due to unstable hemoglobins have a lower spectrin and ankyrin content of membrane, an increase of the proportion of spectrin monomers, a decreased binding of spectrin to protein 4.1 and actin, an alteration of ankyrin structure decreasing the binding of normal spectrin to inside out vesicles of cells containing Heinz bodies (41, 42). Shinar et al. have recently shown (33) that in β thalassemia intermedia spectrin degradation products were not detectable in cell ghosts by the Western immunoblot of SDS-PAGE after the use of comprehensive antiproteolytic agents. The decrease in membrane spectrin is not specific to β thalassemia or to anemia due to unstable hemoglobin since it is also observed in hereditary spherocytosis (43), acquired disorders (44), and during blood aging in vitro (45) but not during in vivo aging of red cells fractionated by centrifugation (46). The decreased amount of normal ankyrin (34%) in β thalassemic cell ghosts was shown by UT-PAGE which separated ankyrin from spectrin β chain better than SDS-PAGE (6). The loss of ankyrin is similar to that of spectrin and has to be compared with the disappearance of ankyrin from the skeleton of cells containing unstable hemoglobins in which the ankyrin-spectrin association is altered, probably by oxidation (36).

Some proteins are present in increased amounts in β thalassemic cell ghosts. As suggested by Shinar et al. (33), these polypeptides could result from attachment of cytoplasmic proteins, including globin chains or polymers of native or degraded hemoglobin chains (35) and other cytosolic proteins or cross-linked membrane proteins, giving rise to higher molecular weight proteins (5), some of which are cleaved by reducing agents. Binding of globin to the membrane skeleton has been shown during aging of normal red cells in vitro (47, 48) and in oxidation of normal red cells in vitro (49, 50).

**Topology of Sulfhydryl Groups**—We confirm that in non-transfused patients with β thalassemia intermedia the free thiol groups of membrane proteins are reduced by 27% (51, 52). Under the conditions used, 80% of normal membrane thiol groups are reacted with NEM (53). This reduction of thiol groups is not evenly distributed among membrane proteins. It affects primarily the most reactive thiol groups of spectrin (~32%) and ankyrin (~65%), in comparison to normal cells in which ankyrin contains a high proportion of the membrane most reactive thiol groups (Fig. 7). Thiol groups exhibiting low reactivity are also decreased in spectrin and ankyrin. Other unidentified protein fractions have modified reactivity of thiol groups.

**Heterogeneity of Free α Chains**—The present study shows that the free α hemoglobin chains are present in different forms in β thalassemic cells: one soluble and able to combine with β hemoglobin chains and various types of insoluble α chains. One form of insoluble α chain can be removed from membrane by dissociation in detergent. They migrate like α chains of hemoglobin in UT-PAGE which is very sensitive to small changes in protein structure induced by mutations or acetylation of NH₂ terminus. Consequently, these α chains seem to be intact α hemoglobin chains with regard to their globin structure. This hemoglobin chain fraction may be similar to that removed by high ionic strength used to induced inside out vesicles (54). Early studies have shown that the fingerprint of the tryptic peptides of the inclusion bodies were similar to that of normal α chain (55). Another type of α chain is linked to other membrane proteins by stable bounds revealed by the immunoblotting. These globin chains have an apparent molecular weight higher than that observed for free α chains and can be homopolymers of α chains (35) or cross-linked polymers with other proteins including spectrin, by disulfides or other linkages.

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