Photochemical Labeling of the Cytoplasmic Surface of the Membranes of Intact Human Erythrocytes*

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N-(4-azido-2-nitrophenyl)-2-aminoethyl sulfonate (NAP-taurine), a photolabile nitrene precursor, has been shown to permeate the human erythrocyte membrane at 37° but not at 0°. Utilizing this differential permeability, we have loaded intact erythrocytes with NAP-[35S]taurine in the dark at 37°, cooled them to 0°, washed them free of external NAP-[35S]taurine in the dark and cold, and photolyzed them, resulting in labeling of hemoglobin and of proteins on the cytoplasmic surface of the membrane. These experiments complement those previously reported on the labeling of the external surface of the membranes with this reagent (Staros, J. V., and Richards, F. M. (1974) Biochemistry 13, 2720-2726).

Much effort has been invested in recent years in attempts to probe biological membranes with the goal of determining the relative geometry of their constituents. The membrane of the human erythrocyte has been the most frequent subject of such structural investigations. The distribution of the various erythrocyte membrane proteins normal to the plane of the membrane (2-20) and the two-dimensional distribution of those proteins in the plane of the membrane (21-24) have been extensively investigated. These and other aspects of membrane structure have been the subjects of several recent reviews (1, 25-29).

In confronting the question of protein distribution in the dimension normal to the membrane plane, the general approach has been to react the external surface of intact erythrocytes with any of various classes of reagents: nucleophile-specific small molecules (2-7); proteolytic enzymes (6, 8-10); iodine, via lactoperoxidase (11-18); or a photochemically generated nitrene (19, 20). Having identified those membrane proteins accessible to a given reagent on the outer surface of the membrane, the cytoplasmic surface has been probed by reacting leaky ghosts (3-5), by reacting inside-out vesicles (6, 9), by resealing ghosts with lactoperoxidase inside (15-18), and, more recently, by saturating the external reactive amino groups with a nonpenetrating reagent and then reacting the cells with a membrane-permeable analog of that reagent (7). The first three of these methods require that the cells be lysed, a treatment which has been shown, under some conditions, at least, to lead to changes in membrane topology (18, 20). The latter method assumes that no significant topological changes are caused by peramidination of reactive amines. The data presented (7), however, indicate that some reorganization may have taken place, in that the authors report one protein susceptible to proteolysis by pronase in the external medium only after amidination of the membranes. A fifth approach has been to cross-link hemoglobin to membrane proteins in intact erythrocytes (24), a procedure useful for studying the cytoplasmic surface, though not the external surface, of the erythrocyte membrane. We report here a procedure by which the cytoplasmic surface of previously unmodified intact erythrocytes was reacted with the nitrene generated by photolysis of N(4-azido-2-nitrophenyl)-2-aminoethyl sulfonate, a reagent previously used to study the external surface of the erythrocyte (19, 20).

EXPERIMENTAL PROCEDURES

All chemical reagents were of ACS certified grade or better. Water for these experiments was distilled, deionized with a Barnsted-mixed bed resin, and finally redistilled in glass.

N-(4-azido-2-nitrophenyl)-2-aminoethyl sulfonate was synthesized by a modification of the procedure described previously (19): purified [35S]taurine was procured from Amersham/Searle at a specific activity of 500 Ci/mol and was used as received. To 1.2 ml of an aqueous solution containing 50 µmol of [35S]taurine and 0.2 mmol of...
sodium carbonate, pH 9.6, in a 10-ml Erlenmeyer flask containing a Teflon-coated micro stirring bar, was added 0.2 mmol of 4-fluoro-3-nitrophenyl azide in 1 ml of acetone. The flask was sealed with attachment to a nitrogen supply and a mercury bubbler. The flask was flushed for 15 min with wet N₂, sealed to the bubbler, and immersed in an oil bath at 60.5 ± 0.5°C. The reaction was allowed to proceed for 61 hours at this temperature, with constant stirring. The purification of the NAP-[¹⁴C]taurine was accomplished as previously described, but the yield by this method was increased to 30% of the radioactivity eluted from the Amicon column.

The procedures for the preparation of erythrocytes, ghosts, and resealed ghosts and their reaction with NAP-taurine; for sodium dodecyl sulfate polyacrylamide gel electrophoretic separations; and for the staining, autoradiography, and fractionation of those electrophoretic gels were those previously described [19, 20] with two exceptions. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out with a concentration of 0.1% (w/v) sodium dodecyl sulfate in the gels and buffers, in order to separate bands 4.1 and 4.2 [30]. Also, in order to minimize the apparent photooxidation of ghost membrane proteins [19], ghosts preparations were photolyzed with NAP-taurine for 10 min—rather than 20 min, the photolysis time for all other preparations.

Erythrocytes were loaded with NAP-taurine by the following procedure. Cells which had been twice washed in phosphate-buffered saline (0.15 M NaCl, 14 mM sodium phosphate, pH 7.4) were washed twice in an incubation medium consisting of 108 mM NaCl, 5 mM KCl, 30 mM Tris-Cl, 22 mM glucose, and 20 μg/ml of chloramphenicol, pH 7.4. An aliquot, 0.25 ml, of the packed cells was suspended in 0.25 ml of the same incubation medium, but containing 0.7 mM NAP-taurine. The suspension, protected from light, was incubated at 37°C for 18 hours, with very gentle agitation, and was then cooled to 0°C in an ice bath. The suspension was diluted with 20 ml of phosphate-buffered saline, containing 1% (w/v) bovine serum albumin, Fraction V (Sigma), at 0°C. The cells were pelleted by centrifugation at 3°C at 12,000 × g, for ≤ 1 min at 0°C, in an SS-34 rotor of a Sorvall RC-5B refrigerated centrifuge. The cells were washed by resuspension and centrifugation in the albumin-containing buffer nine additional times, followed by two or three washes under the same conditions in phosphate-buffered saline containing no albumin. The final pellet was either resuspended in 0.25 ml of phosphate-buffered saline at 0°C for photolysis and then handled as previously described or was resuspended in 10 ml of the same buffer at 0°C for measurement of NAP-taurine efflux, as described below.

Efflux of NAP-taurine was measured by sampling a 10-ml suspension of NAP-[¹⁴C]taurine-loaded erythrocytes prepared as described above. From each 1.0 ml sample, 0.30 ml was reserved to measure total NAP-taurine. The remainder was centrifuged quickly, and 0.30 ml of the supernatant was removed for counting. To each of the reserved 0.30-ml samples was added 0.30 ml of 6% perchloric acid. The samples were mixed and centrifuged in a clinical centrifuge, and 0.50 ml of the supernatant was dried at 31°C and counted in toluene-liquid scintillator.

**RESULTS**

The relative permeabilities to NAP-taurine of the membranes of intact erythrocytes at 0°C and at 37°C is demonstrated by the efflux data of NAP-[¹⁴C]taurine-loaded cells presented in Fig. 1. There is no measurable efflux of NAP-taurine at 0°C; while at 37°C, NAP-taurine is rapidly lost from the cells. These data strongly support the previous finding that NAP-taurine does not permeate the erythrocyte membrane at 0°C [19].

Erythrocytes loaded with NAP-[¹⁴C]taurine and washed with buffer containing bovine serum albumin were photolyzed, and the cells were subsequently lysed and the membranes isolated, purified, and subjected to electrophoresis in sodium dodecyl sulfate polyacrylamide gels, which were cut into 1-mm fractions and counted. In order to distinguish what fraction, if any, of the consequent labeling might have resulted from NAP-[¹⁴C]taurine adsorbed to the outer face of the cells and not removed by washing, a control was run in parallel with the above experiment. An aliquot of erythrocytes was incubated overnight as described, but in incubation medium without NAP-taurine. After the suspension had cooled to 0°C, the cells were washed once in incubation medium and then resuspended at 0°C in the same medium containing 0.7 mM NAP-[¹⁴C]taurine. The suspension was agitated gently in an ice bath for ~1 hour and was then washed, photolyzed, and otherwise worked up in parallel with the experimental sample. The radioactivity profiles produced by this experiment and control are shown together in Fig. 2. Essentially no labeling resulted from residual NAP-taurine adsorbed to the outer face of the membrane.

An aliquot of the same labeled membrane preparation that was used to produce the upper curve of Fig. 2 was subjected to electrophoresis in a second sodium dodecyl sulfate polyacrylamide gel. The gel was fixed, stained, sliced longitudinally, dried, and autoradiographed. A densitometer trace of the
autoradiograph and photographs of the autoradiograph and the stained gel slice from which it was made are shown in Fig. 3.

The Coomassie blue stain associated with globin in Fig. 3 indicates that very little hemoglobin was isolated with the membranes. Yet the $^{35}$S label moving with the globin chains is the dominant feature in Figs. 2 and 3. The predominantly labeled membrane protein is Band 3. Bands 1 and 2 (spectrin) are labeled, but only very lightly. Bands 2.1, 2.2, 2.4, and 2.5 are labeled, but there is no indication of labeling of Band 2.6. Band 4.2 is labeled, but Band 4.1 carries essentially no label. Band 6 is labeled. Band 5 is obscured by globin trimer.

In order to test for changes in topology which may have occurred with the incubation procedure, cells which were incubated in medium containing no NAP-taurine—and others, which were incubated in medium containing 0.7 mM nonradioactive NAP-taurine—were labeled on the exterior by the method previously described (19), and the electrophoretic patterns of the proteins from those preparations were compared with that obtained from labeled washed cells which had not been incubated. The results are shown in Fig. 4. The patterns of Coomassie blue-stained protein of these samples are virtually indistinguishable as are the corresponding autoradiographs.

In order to compare previous results on the external, NAP-taurine labeling of resealed ghosts with other results presented here, resealed ghosts were prepared at a hemolytic ratio of 20, labeled as before (20) and subjected to electrophoresis in the 0.1% sodium dodecyl sulfate gel system. Fig. 5 shows that the previously reported accessibility of a component of Band 4 to NAP-taurine in the external medium is due to the accessibility of Band 4.2, rather than of Band 4.1 or of both. It should be further noted from Fig. 5 that a small quantity of hemoglobin was isolated along with the membranes after the final lysis, and that a globin band does appear in the Coomassie blue-staining gel slice. No radioactivity, however, is associated with this globin band, supporting the earlier contention (20) that resealed ghosts, like intact cells, are impermeable to NAP-taurine at 0°C on the time scale of these experiments.

Leaky ghosts were prepared from fresh erythrocytes as previously described (19). Leaky ghosts were also prepared (in the dark) from intact cells which had been incubated at 37°C with 0.7 mM nonradioactive NAP-taurine in order to test whether or not the procedure used to load the cells with NAP-taurine had any effect on the labeling pattern. Both samples were then photoconverted in the presence of NAP-$^{35}$S taurine. Electrophoretic patterns of these two membrane preparations, Fig. 6, show no significant difference in either the banding pattern of Coomassie blue-staining proteins or in labeling pattern. In both of the preparations Band 4.1 carries...
very little label. Thus, Bands 1, 2, 4.1, and 5 (19) carry less label relative to stain than do the other Coomassie blue-staining components.

**DISCUSSION**

The unreactivity of NAP-taurine before photolysis, combined with the sharp change in the permeability of the erythrocyte membrane toward that molecule from 0° to 37° has permitted the experiment of modifying the cytoplasmic surface of the membrane of an intact, previously unmodified cell. A possible explanation for the permeability of NAP-taurine at body temperature is suggested by a comparison of our data with that of Cabantchik and Rothstein (31-33) on the affinity labeling of the anion channel of the erythrocyte. That NAP-taurine penetrates the erythrocyte membrane at 37° via the anion channel is suggested by the observation that the predominantly labeled membrane protein in our experiments is Band 3. This band includes a polypeptide that has been identified as a subunit (23) of the anion channel by affinity labeling with aryl sulfonates (31, 32) including NAP-taurine (33). This protein has recently been isolated and characterized by Ho and Guidotti (34). That NAP-taurine is bound by this protein (though it is not a photoaffinity reagent for it by the definition proposed by Ruoho et al. (35)) is further supported by the following observation (data not shown). Photolyzed samples of cells prepared as in the control experiment, **Fig. 6**a, were washed less thoroughly with albumin-containing buffer. Band 3 was the last band to be washed free of NAP-taurine.

As discussed previously (19), one would expect spectrin (Band 1 + Band 2) to be labeled from the cytoplasmic side of the membrane. While NAP-taurine inside the cell does react with spectrin, the extent of labeling is low. Since spectrin reacts readily with a number of other low molecular weight reagents, it is hard to imagine any strictly steric effect in the NAP-taurine reaction. Extensive reaction occurs with alkyl imidates of comparable size (7, 23, 24) which are largely cationic. NAP-taurine is an anion. A possible explanation may involve differences in the net charges on the various proteins and consequent differences in the surrounding ionic atmospheres. In highly charged systems (i.e. micelles) reagent ion exclusion or inclusion can have enormous effects on the observed rates of bimolecular reactions. Whatever the explanation in the present case, it is clear that the relative labeling patterns with the various reagents are significantly different.

In the future such differences may become useful as indicators of structural detail.

Another observation of the same nature concerns the Band 4.1 protein. This protein is essentially unlabeled by NAP-taurine in intact cells or resealed ghosts, and it is only lightly labeled in leaky ghosts. Yet, Wang and Richards (23, 24) have shown that this protein is readily cross-linked to spectrin in intact cells and in leaky ghosts, and that it can be cross-linked to hemoglobin in intact cells. Although the lower resolution of sliced gels leads to some uncertainty, in their studies with alkyl imidates Whiteley and Berg (7) conclude that this component is labeled from both the inside and outside of the membrane. Again differential reactivity with reagents of opposite charge may be indicated.

The hemoglobin polymerization observed in this work is probably a parallel side reaction accompanying the insertion process which is of principal interest for labeling. Since radicals are produced in the reactions of nitrenes, it is possible that the polymerization is due to radical recombination between adjacent peptide chains. Such a problem would be expected to be particularly severe in very concentrated solutions such as the cytoplasm in the erythrocyte.

The Band 4.2 protein is clearly labeled by NAP-taurine only from the inside of intact cells, but it is labeled from the outside of resealed ghosts. Though the accessibility of this component changes with respect to the dimension normal to the plane of the membrane with lysis and resealing, it appears to maintain its apparent near neighbor geometry in the plane of the membrane in intact cells and ghosts (23, 24). However, an accessibility change such as we have observed could result from a rather subtle structural change, such as the establishment of a water channel part way through the membrane. In addition, both the NAP-taurine labeling and the above cross-linking techniques are nonquantitative by design; polypeptide chains which maintain their apparent proximity to the Band 3 protein in ghosts may be topologically distinct from those which are labeled from the outside of resealed ghosts.

As previously discussed (19) membrane proteins may be operationally divided into four classes: I, exterior; II, interior; III, trans-membrane; and IV, buried. It appears that all, or virtually all, erythrocyte membrane proteins are of Classes II or III. Though Band 4.1 is not labeled by NAP-taurine from the inside or the outside in intact cells, other evidence, cited above, argue strongly for its being of Class II rather than Class IV. Of those proteins un obscured by other bands on the gels, only Band 2.6 is clearly labeled from the outside but not from the inside. Therefore, this protein remains a candidate for Class I (exterior), but negative evidence in experiments of this type is weak. While several proteins, including those of Bands 2.1, 2.2, 2.4, 2.5, and 3, can be assigned to Class III, strong evidence that a given polypeptide spans the membrane is limited to component(s) of Band 3. Bender et al. (8) showed that essentially all of Band 3 is cleaved by pronase treatment of intact erythrocytes, and Bretscher (4) demonstrated that new
sites on undegraded Band 3 component(s) were made accessible by lysis. These data were interpreted as indicating that Band 3 polypeptide spans the membrane. However, in order to make this interpretation, one must assume that the detailed structure of the membrane is the same after lysis as it is in the intact cell. Recent evidence (18, 20) suggests that this assumption may not always be valid. However, since we were able to label Band 3 on the cytoplasmic surface of previously unmodified cells, our data combined with the earlier pronase digestion data (8) strongly indicate that some component(s) of band 3 do span the membrane, a result not surprising in light of the data linking Band 3 to anion transport (31-34).

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