Specific Modification, Isolation, and Partial Characterization of an Erythrocyte Membrane Protein*

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SUMMARY

Treatment of erythrocytes or their isolated membranes with radioactive iodoacetate results in the specific modification of one of the membrane proteins, as determined by acrylamide gel electrophoresis in sodium dodecyl sulfate. The reactive groups of this protein are almost completely blocked before any of the other major membrane proteins react. The reaction with N-ethylmaleimide does not show a similar specificity of labeling, indicating a considerable degree of reagent specificity for the reaction. The specifically labeled protein was extracted from the membrane with sodium chloride and further purified by chromatography on Sephadex G-200 in sodium dodecyl sulfate. This preparation shows a single band on acrylamide gel electrophoresis in sodium dodecyl sulfate with a molecular weight of 35,000. The labeled amino acid was identified as cysteine from its elution position on ion exchange chromatography.

In the present study sulfhydryl reactivity has been investigated at three levels: the intact erythrocyte, the resealed ghost, and the isolated membrane. An enhanced specificity of reaction with membrane sulfhydryl groups can be achieved with low concentrations of iodoacetate, limiting reaction almost exclusively to a single protein. This observation, coupled with the isolation and partial characterization of this protein, offer the possibility of a dual approach to understanding some of the problems of membrane protein chemistry.

EXPERIMENTAL PROCEDURES

Materials and Methods—Human blood was obtained from the Dallas Community Blood Bank and used within 1 week of the withdrawal date. Chemicals for acrylamide gel electrophoresis were obtained from Eastman or Canaleo and were the highest purity available from these sources. Radioactive iodoacetic acid (14C, specific activity 13.9 mCi per mmole; 3H, 81.5 mCi per mmole) was obtained from New England Nuclear, and N-ethylmaleimido (2.1 mCi per mmole) was obtained from Amer sham-Searle. SDS, bovine serum albumin, &-galactosidase, catalase, chymotrypsinogen, ovalbumin, and cytochrome c were all products of Sigma. Columns and chromatographic supports were obtained from Pharmacia or Bio-Rad.

Protein was determined by the procedure of Lowry et al. (10), and hemoglobin as cyanemethemoglobin (11). Radioactivity was measured by scintillation counting in either Bray’s solution (12) or in toluene-ethanol. Samples containing hemoglobin were corrected for quenching using a standard quenching curve for known hemoglobin concentrations. Radioactivity measurement were corrected for machine efficiency and quenching by external standardization. Samples for amino acid analysis were hydrolyzed for 22 hours at 110° (13). Amino acid analyses were performed on a Beckman 120 C amino acid analyzer using a three buffer, single column elution system on the long column. Cysteic acid analyses were performed by the method of Hirs (14), and typtophan was determined after hydrolyses with p-toluene-sulfonic acid (15).

Preparation of Membranes—Red cells were washed with 0.9% NaCl solution to remove plasma and buffy coat. For preparation of resealed ghosts (16) 1 volume of packed red cells was hemolyzed in 10 volumes of ice-cold 10 mm Tris buffer (pH 7.4).

The abbreviations used are: SDS, sodium dodecyl sulfate; NEM, N-ethylmaleimide; IAA, iodoacetic acid.

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2 P. Guire, unpublished observations.
at 4°C for 10 min. This suspension was then treated with 1 volume of a mixture of 1.42 M KCl and 0.28 M NaCl and kept at 25°C for 15 min. The resealed ghosts were washed at 4°C with 10 volumes of NaCl solution, then with Krebs-Ringer solution. For the preparation of erythrocyte membranes a modification of the procedure of Dodge et al. (17) was used. One volume of packed red cells was hemolyzed in 10 volumes of 7 M sucrose (pH 7.4) at 4°C and washed three times with 10 volumes of the same buffer. The ghosts prepared by this procedure contain less than 1% of the original cellular hemoglobin. Some variations in the hemoglobin content of modified membranes were noted as described in later sections.

Labeling of Erythrocyte Membranes of Red Cells, Resealed Ghosts, or Isolated Membranes—Washed cells, resealed ghosts, or isolated ghosts were allowed to react for 90 to 120 min at 37°C with [3H]-iodoacetate (0.25 mM, 4 μCi) or N-ethyl[4C]maleimide (1.65 mM, 10 μCi) in Krebs-Ringer buffer (pH 7.4) at a cell or ghost concentration equivalent to a red cell hematoctit of 30 (1.6 mg per ml of membrane protein). The intact cells and resealed ghosts were washed with Krebs-Ringer buffer, then hemolyzed as described above. The isolated ghosts were washed with Krebs-Ringer buffer, then with 7 M phosphate (pH 7.4) before being prepared for electrophoresis.

For the double labeling experiment, 1.0 ml of packed red cells was suspended in 2.0 ml of Krebs-Ringer buffer (pH 7.4) and allowed to react with [3H]-iodoacetate (0.59 mM, 80 μCi) at 35°C for 60 min. The labeled cells were washed and hemolyzed as described previously. The isolated ghosts were then reacted with [3H]-iodoacetate (0.75 mM, 10 μCi) in Krebs-Ringer buffer at 35°C for 60 min. After being washed with 7 M phosphate buffer (pH 7.4), the ghosts were prepared for electrophoresis.

**SDS Acrylamide Electrophoresis—**For electrophoresis, 200 μl of packed membranes (0.7 to 1.0 mg of protein) were dissolved by adding 100 μl of a freshly prepared solution of 4% SDS and 2.5% mercaptoethanol and incubating overnight at room temperature. Glycerol (to 15 to 90%) was added to the sample before loading directly onto the gels. For lyophilized samples, added amounts were dissolved in 2% SDS and 2.5% mercaptoethanol in 20 mM phosphate at pH 7.4 by incubating overnight at room temperature. The gels were prepared and run according to previously described procedures (18, 19) using a 5% acrylamide concentration and 10- to 13-cm gels. For radioactive samples, one gel was stained overnight at room temperature with 0.05% Coomassie blue in 10% methanol and 7% acetic acid. The gel was destained in 7% acetic acid in a Hoefer destainer. The second gel was sliced immediately after the electrophoresis run with a homemade gel slicer (20). The slices were extracted and counted by a modification (21) of the procedure of Basch (22). The gel patterns observed by staining did not differ significantly from those reported previously for human erythrocyte membranes (21, 23-25), except for the higher resolution of bands achieved by using the longer gels and a lower SDS concentration in the gel (26). The molecular weights of the membrane proteins were estimated from a plot of the logarithm of molecular weight versus migration distance for a series of standard proteins: hemoglobin, chymotrypsinogen, catalase, bovine serum albumin, and β-galactosidase. Proteins were dissolved in SDS and reduced as described for membranes.

**Gel Filtration in SDS—**Membrane samples or lyophilized fractions were dissolved in a solution of 1% SDS, 0.05 M phosphate, and 0.02% sodium azide (pH 7.0) and reduced with 1% mercaptoethanol. Chromatography was performed on Bio-Gel P-100 (3.5 X 80 cm), Sephadex G-200 (2.5 X 90 cm), or Sepharose 4B (2.5 X 90 cm) at room temperature using the SDSPHosphate-amine system as the elution buffer. Fractions for electrophoretic or amino acid analysis were dialyzed at room temperature for 2 to 3 days against 40% methanol to remove SDS, against distilled water at 4°C overnight, and lyophilized. The molecular weight of the isolated membrane protein was estimated from its elution position on G-200 in comparison to a standard curve prepared using bovine serum albumin, ovalbumin, and cytochrome c.

**Identification of Labeled Amino Acid—**Samples of the labeled material (membranes or purified protein) were hydrolyzed at 110°C for 22 hours and applied to the long column of a Beckman 120 C amino acid analyzer. The column was eluted using a three buffer system, and fractions were collected. Samples of 0.5 ml were counted in Bray's solution. The elution system was calibrated by observing the elution positions of C-labeled serine, alanine, and tyrosine. The elution position of any amino acid could then be calculated from its known elution volume in the standard ninhydrin analytical system.

**RESULTS**

**Specificity of Reactions—**To study the reactivity of the various proteins in the membrane toward sulfhydryl reagents, erythrocyte membranes in three forms, the intact cell, the resealed ghost, and the isolated membrane, were subjected to reaction with radioactive iodoacetate acid or N-ethylmaleimide. Both reagents penetrated the membrane, as shown by radioactivity incorporated into the hemolysates from the red cells and resealed ghosts. The amounts of radioactivity incorporated into the membrane and membrane fractions were dependent on the ratio of reagent to cells and the efficiency of removal of the intracellular proteins from the membrane. Prior labeling of cells and resealed ghosts caused a slightly increased retention of the intracellular proteins after hemolysis and washing of the membranes. This effect was variable for different blood samples, but it did not affect the results except as noted. The specific activities of the iodoacetate-labeled hemolysate protein and membrane protein were 2.1 X 10^6 dpm per mg of hemoglobin and 4.9 X 10^6 dpm per mg of membrane protein, respectively, for a typical experiment. Since the carboxymethyl groups are not equally distributed among all of the membrane proteins, this shows that there is considerably higher incorporation into the labeled membrane proteins than into hemoglobin. This suggests a higher reactivity for the membrane protein reactive functional groups, since both membrane groups and hemoglobin groups are in competition for the reagent.

The relative reactivities of the individual membrane proteins can be determined by fractionating the membrane by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and determining the radioactivity of the various protein bands by slicing and counting the gel. The results of a typical iodoacetate labeling experiment are shown in Fig. 1. Surprisingly, only a single band of the fractionated membrane contains a significant amount of the radioactivity incorporated into the membrane when the intact red cell is labeled. The resealed ghost and ghosts, which show this band as the major labeled species, but other proteins have been labeled more heavily in these cases. At the top
of Fig. 1 is shown a schematic representation of the protein distribution as determined by staining with Coomassie blue. The superimposed molecular weight scale permits us to estimate a value of about 40,000 for the labeled band. This band corresponds to Component VIII by our previous system of labeling the erythrocyte membrane proteins shown at the top of Fig. 2 (26).

The specificity of the reaction is not nearly so great for N-ethylmaleimide labeling, as shown in Fig. 2. This reagent labels all of the major proteins of the membrane. For the ghost, the amount of label incorporated into each band roughly parallels the staining intensity of the band with Coomassie blue. Component VIII, which is heavily labeled in the iodoacetate experiment, is not specifically labeled by N-ethylmaleimide under these conditions. Fig. 2 also shows the increased retention of intracellular proteins observed after labeling the resealed ghost. Both hemoglobin and Component X were observed in significant quantities by staining in the case of the labeled resealed ghost, even though they were virtually absent from the staining patterns in the cases of the red cell, which was less heavily labeled, and the ghost, which was washed before labeling.

The foregoing results show that only one band of the erythrocyte membrane protein pattern is specifically labeled by iodoacetate if the red blood cell is reacted. The reaction specificity is decreased in the ghost and abolished by changing the reagent to N-ethylmaleimide. To investigate the reaction specificity in more detail, the concentration dependence of the reactions with the two reagents was studied with isolated ghosts (Fig. 3). Several interesting features of the reaction system were discovered.

(a) The number of N-ethylmaleimide groups incorporated was considerably greater (15- to 20-fold) than the number of carboxymethyl groups for equivalent reagent concentrations. (b) Incorporation of NEM groups approached saturation at 0.5 mM, but IAA incorporation was still increasing in a linear fashion. (c) The specificity of the IAA reaction was dependent on reagent concentrations (Fig. 4). At the lowest reagent concentration used, Component VIII was labeled almost exclusively, indicating that the specificity difference observed between the ghost and red cell was a function of effective reagent concentration and not due to a difference in membrane properties. (d) The specificity

![Fig. 1. Radioactivity profile of [14C]iodoacetate labeled membranes from treated erythrocytes, resealed ghosts, and ghosts. The three types of samples were labeled at a membrane protein concentration of 1.6 mg per ml with 0.25 mM [14C]iodoacetate in Krebs-Ringer phosphate (pH 7.4). Membranes were isolated by the procedure of Dodge et al. (17), solubilized in SDS, and fractionated by electrophoresis in 0.1% SDS on 5% acrylamide gels. Duplicate gels were sliced and counted for radioactivity and stained with Coomassie blue. The diagram at the top of the figure shows the staining pattern observed, which was essentially identical for all of the gels. The molecular weight distribution for the protein bands is also shown at the top of the figure.]

![Fig. 2. Radioactivity profile for N-ethyl[14C]maleimide-labeled membranes from treated erythrocytes, resealed ghosts, and ghosts. The samples were labeled as described in Fig. 1, at a NEM concentration of 1.65 mM. Membrane isolation and electrophoretic analyses were performed as described in Fig. 1. At the top of the figure is shown the Coomassie blue staining pattern and a numerical assignment of band areas described previously (26). Component X and hemoglobin were present to a significant extent only in the membranes from labeled resealed ghosts.]

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FIG. 3. Concentration dependence of iodoacetate and N-ethylmaleimide reactions with isolated erythrocyte membranes. Membranes were labeled as described in Figs. 1 and 2 with reagent concentrations from 50 to 400 nM. Labeled membranes were washed, counted, and analyzed for protein by the Lowry method (10).

![Graph 1](image1)

**Fig. 3.** Concentration dependence of iodoacetate and N-ethylmaleimide reactions with isolated erythrocyte membranes. Membranes were labeled as described in Figs. 1 and 2 with reagent concentrations from 50 to 400 nM. Labeled membranes were washed, counted, and analyzed for protein by the Lowry method (10).

![Graph 2](image2)

**Fig. 4.** Concentration dependence of specificity of iodoacetate labeling of erythrocyte membranes. Conditions for labeling and electrophoresis are described in Figs. 1 and 2.

![Graph 3](image3)

**Fig. 5.** Sepharose 4B fractionation of protein from labeled resealed ghosts. Membranes from labeled resealed ghosts were separated into protein and lipid fractions by chromatography on Bio-Gel P-100 in 1% SDS. The protein fraction was dialyzed to remove SDS and lyophilized. The sample was redissolved in 1% SDS-0.02% sodium azide-0.05 M phosphate (pH 7.0) and chromatographed on Sepharose 4B (column, 2.5 x 90 cm) with the same buffer. Protein (A$_{280}$, ---) and radioactivity (cpm, -- - - - ) were monitored on 3-m1 fractions. Fractions denoted by the black bar were collected for SDS acrylamide electrophoresis and identification of the labeled amino acid.

of the NEM reaction was not dependent on reagent concentration over the range used. However, since the number of NEM groups incorporated at the lowest concentration of this reagent used for these experiments was still about 4-fold greater than the IAA groups incorporated at its highest concentration, a further experiment was performed at a lower NEM concentration. In this case only 0.5 n mole of NEM was incorporated per mg of membrane protein. Since the level of radioactivity precluded radioactivity analysis by acrylamide gel electrophoresis, the membranes were fractionated by column chromatography in SDS on Sepharose 4B. Comparison of the radioactivity and absorbance profiles gave no indication of an enhanced specificity of reaction even at these low concentrations. Therefore, the reaction of N-ethylmaleimide must be considerably less specific than the reaction with iodoacetate and does not show a strong concentration dependence of specificity.

In order to show clearly that Band VIII corresponded to the labeled component, a sample of membranes from labeled resealed ghosts was subjected to electrophoresis. The gels were stained with Coomassie blue, and the individual bands were sliced from the gels and counted. Bands I and VIII contained the bulk of the radioactivity, in agreement with the radioactivity profile of the membranes from labeled resealed ghosts (Fig. 1).

**Partial Characterization of Labeled Band**—The labeling and electrophoresis studies indicate that a single protein of the erythrocyte membrane can be heavily labeled with iodoacetate. However, because of possible anomalies which could arise in the labeling or electrophoresis experiments, it was desirable to use other methods to verify the nature of the labeled component. Several experiments were performed to confirm that the labeled component was not loosely bound to the membrane. Labeled membranes were subjected in separate experiments to repeated washings with 7 mM phosphate (pH 7.4), dialysis at 4°C against distilled water (24 hours), and extraction with 8% mercaptoethanol at pH 8.0 at room temperature (18 hours). In none of these cases was the radioactive profile of the membranes altered.

Additional characterization experiments to show the protein nature of the labeled resealed ghosts were performed on membranes isolated from IAA-labeled resealed ghosts. Membranes were solubilized in 1% SDS and chromatographed on a Bio-Gel P-100 column, a procedure which separates the membrane protein from the lipid. Essentially all of the radioactivity (>95%) was eluted in the protein peak at the excluded volume of the column. Combined fractions of the protein peak were subjected to a second chromatographic analysis on a Sepharose 4B column in 1% SDS. The protein and radioactivity profiles of this peak are shown in Fig. 5. Fractions were collected from the major

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radioactive peak for electrophoretic analysis and identification of the labeled amino acid. SDS electrophoresis of this material showed the presence of several protein bands stained by Coomassie blue. Only one of these (corresponding to the migration position of the Component VIII) contained radioactivity.

Identification of the labeled amino acid was achieved by chromatography on the long column of a Beckman 120 C amino acid analyzer. Two radioactive peaks were eluted. The major peak (90%) corresponded in position to carboxymethylcysteine. The minor peak eluted slightly after the position of cysteic acid and did not correspond to any known amino acid or known carboxymethyl derivative. Samples of unfraccionated membrane protein from labeled ghost or resealed ghosts were also analyzed for their labeled amino acids. These showed a peak corresponding to the position of carboxymethylhistidine as well as carboxymethylcysteine.

Purification of Labeled Protein—Previous studies (26) from our laboratory have shown that Component VIII can be extracted from the isolated membrane by dialysis against 0.3 M sodium chloride, 0.5 mM EDTA, and 5 mM mercaptoethanol at pH 9.5. An even more specific procedure, using a brief exposure to 0.5 M sodium chloride, has been reported by Fairbanks et al. (24). Comparison of electrophoretic patterns suggested that the two procedures extracted the same protein. Therefore the sodium chloride extraction procedure was investigated as a means of purifying the labeled component. Membranes from IAA-labeled red cells were extracted according to the procedure of Fairbanks et al. (24). Because our hemolysis procedure did not remove all of the hemoglobin from the membranes, it was not possible to determine quantitatively the percentage of membrane protein extracted. However, it was shown that 30% of the membrane radioactivity was extracted. SDS acrylamide electrophoresis showed two protein bands (Fig. 6) corresponding to hemoglobin and Component VIII, with essentially all of the radioactivity present in the latter. For purification of sufficient quantities of protein for analytical purposes, samples of labeled and unlabeled sodium chloride extracts were mixed, solubilized in SDS, and subjected to chromatography in 1% SDS on Sephadex G-200. The elution profiles for protein, heme, and radioactivity are shown in Fig. 7. The elution position of the labeled protein corresponds to a molecular weight of 42,000. Because heme and globin are dissociated in the SDS solution, their peaks do not coincide, although they fortuitously overlap because of the aggregate size of the heme-SDS complex. It is interesting to note that the globin is almost completely devoid of radioactivity. The fractions indicated were combined for further analysis. SDS electrophoresis of the labeled protein showed only a single band for both protein stain (Fig. 6) and for radioactivity, indicating that the polypeptide was pure by this criterion. The amino acid analysis of the purified Component VIII is shown in Table 1. It is significantly different from the analyses for whole erythrocyte membranes (26) and for spectrin, the erythrocyte membrane protein fraction which is extracted from the membranes with EDTA (27). Of particular interest was the half-cystine content of Component VIII. Performic acid oxidation and analysis for cysteic acid gave a value of 1.0 mole per cent, corresponding to 2.9 moles of half-cystine per mole of protein. The combined fractions of the second peak from Sephadex G-200 were also subjected to amino acid analysis to confirm its identification as globin. A sample of the purified Component VIII was hydrolyzed for identification of the labeled amino acid. Car-

![Fig. 6. SDS acrylamide electrophoresis of purified fractions of membrane protein. Gels contain (left to right): isolated erythrocyte membranes, sodium chloride extract, purified Component VIII after Sephadex G-200 chromatography, and Fraction II (globin) from Sephadex G-200 chromatography.](http://www.jbc.org/)

![Fig. 7. Purification of labeled Component VIII by chromatography on Sephadex G-200 in 1% SDS. The labeled component was extracted from erythrocyte membranes with 0.5 M sodium chloride. The extracts were dialyzed at 4° against distilled water to remove salt and lyophilized. The sample was dissolved in buffered 1% SDS (see Fig. 5) and chromatographed on Sephadex G-200. Fractions of 3 ml were collected and analyzed for protein (A280), heme (A412), and radioactivity (cpm). Fractions denoted by the black bars were combined for electrophoresis and amino acid analysis.](http://www.jbc.org/)
Amino acid composition of purified Component VIII

| Amino acid | Mole % | Residues per molecule |
|------------|--------|----------------------|
| Lysine     | 8.36   | 23.5                 |
| Histidine  | 3.41   | 9.0                  |
| Arginine   | 3.32   | 9.3                  |
| Aspartic acid | 10.98 | 30.9                |
| Threonine* | 5.87   | 16.5                 |
| Serine     | 6.31   | 17.8                 |
| Glutamic acid | 7.66  | 21.0                 |
| Proline    | 3.95   | 11.1                 |
| Glycine    | 10.36  | 29.2                 |
| Alanine    | 10.29  | 29.0                 |
| Half-cystine| 1.04  | 2.9                  |
| Valine     | 8.27   | 23.5                 |
| Methionine | 2.07   | 5.8                  |
| Isoleucine | 5.52   | 15.5                 |
| Leucine    | 7.12   | 20.0                 |
| Tyrosine   | 1.90   | 5.3                  |
| Phenylalanine | 3.77 | 10.7                 |
| Tryptophan | 4.77   | 10.0                 |

* Values obtained from 22-hour hydrolysis with 3 N p-toluenesulfonic acid (15).

** Values obtained as cysteic acid by method of Hirs (14) and corrected for oxidation losses by comparison with ribonuclease samples hydrolyzed under identical conditions.

a Calculated based on molecular weight of 35,000.

** Values obtained from 22-hour hydrolysis with 3 N p-toluenesulfonic acid.

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The reaction of iodoacetate with the erythrocyte membrane shows an unusual specificity, in which one of the 10 or 12 major membrane proteins can react to give virtual saturation of its reactive groups before any of the other proteins react to a significant extent. This specific reaction occurs almost completely at the sulfhydryl groups of this protein, although other protein side chains were shown to react at higher reagent concentrations. A second sulfhydryl reagent, N-ethylmaleimide, shows completely different behavior, labeling the membrane proteins rather nonspecifically, under the conditions used, in approximate proportion to the quantity of protein in the membrane. Thus the reactivity observed appears to be reagent-specific as well as protein-specific. The labeling patterns with N-ethylmaleimide observed in the current study are similar to those of Lenard (9) in that the high molecular weight protein spectrin is most heavily labeled. However, we also observed heavier labeling of the proteins in the 100,000 molecular weight region than were reported previously. The discrepancy between these results may derive from differences in reaction conditions or in prior treatments of the membranes. Bellhorn et al. (28) have noted that the sulfhydryl content of the erythrocyte membrane decreases under certain conditions.

The observation that the number of N-ethylmaleimide groups incorporated into the membrane approaches the saturation condition at the highest reagent concentrations permits us to estimate the number of reactive sulfhydryl groups in the isolated membrane at 20 mmollg of membrane protein. This corresponds to a total of 2.2 reactive cysteines per 100 amino acid residues (assuming an average molecular weight of 110 for an amino acid), which compares favorably with a value reported previously from modification studies with high concentrations of iodoacetamide (28). A value of $10^{-17}$ moles of reactive cysteine residues per cell membrane was calculated from these data, based on the recovery of 5.0 mg of membrane protein per ml of packed red cells and the assumption of $10^{10}$ packed cells per ml.

The identification of the iodoacetate-labeled protein as Component VIII permitted a simple purification, based on a sodium chloride extraction reported earlier by Fairbanks et al. (24). The efficiency of extraction in our work appears to be lower than that obtained earlier, but this may result from variations in the treatment of the membranes during the labeling and hemolysis. Alternatively, the modification of the protein sulfhydryl groups...
may effect its extractability. The purification of this protein represents one of the few examples of a membrane protein which has been purified to this state of homogeneity. However, although the protein is homogeneous by SDS acrylamide electrophoresis, the possibility that more than one polypeptide chain is present in this preparation cannot be completely ruled out. The specificity of the extraction and of the labeling do suggest that all of the isolated components must be closely related, if multiple components indeed exist. Further studies of the properties of this protein will be necessary to determine its exact constitution.

The purification of Component VIII presents an excellent opportunity to study some of the properties of a membrane protein which might be relevant to its function. Consideration of the amino acid composition is not particularly revealing, since it does not show an unusually high content of hydrophobic residues or any other anomalous features. The composition is also quite different from spectra and other "ectrin-type structural proteins" (29). The question of whether Component VIII is truly a structural component of the membrane is an intriguing problem. In addition to its unique extraction and reaction behavior, this protein has been shown to be resistant to proteolysis (24) and to be released from isolated membranes by proteolysis (24). Incorporation of ATP into resealed ghosts before labeling with iodoacetate also results in a marked decrease in the retention of Component VIII upon washing the membranes in hypotonic buffers. The case of removal of this protein from the membrane under various conditions may indicate a peripheral location at one of the membrane surfaces, probably the interior surface, since labeling studies with nonpenetrating membrane reagents do not show any incorporation into this component (30, 31). The variations in extractability, digestibility, and reactivity observed for the various erythrocyte membrane proteins suggest that there may be several levels of structure at which proteins can be associated with the membrane. There are no obvious explanations available at present to account for the specificity of the iodoacetate reaction. This behavior could result either from the environment of the sulfhydryl groups in the protein structure of Component VIII or from the environment imposed upon the protein by its incorporation into the membrane. It is interesting to note that the hemoglobin which is not removed from the membrane during hemolysis and washing does not contain any iodoacetate, suggesting that its reactivity is affected by association with the membrane. Further reactivity studies will be necessary to relate the membrane iodoacetate labeling to membrane or protein structural and functional parameters. The uniqueness of the reaction suggests that it will be a useful tool for obtaining insights into the complex nature of membrane functional organization.

Note Added in Proof—Enzyme activity measurements and iodoacetate inhibition studies indicate that the primary iodoacetate-labeled component of the erythrocyte membrane is a subunit of glyceraldehyde 3-phosphate dehydrogenase. This discovery is important in that it gives us the first identification of a functional activity which is associated with the erythrocyte membrane in significant quantity, permits an estimation of the quantity of a particular enzyme associated with the membrane in terms of the amount of protein present, and establishes a known standard to which iodoacetate inhibition of other membrane activities can be compared.

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