(^{51}CR) SODIUM CHROMATE INCORPORATION INTO THE SOLUBLE PROTEIN FRACTION OF THE HUMAN ERYTHROCYTE: BINDING NOT ASSOCIATED WITH THE HEMOGLOBIN MONOMERIC SUBUNIT

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SUMMARY

The distribution of radioactive chromium on proteins isolated in the soluble fraction of the human erythrocyte was studied utilizing sodium dodecyl sulfate polyacrylamide gel electrophoresis. The radioactive label was associated with the hemoglobin monomeric subunit and, unexpectedly, with another soluble fraction protein. This protein was approximately 6% of the total isolated protein, contained approximately 20% of the radioactivity, and had an estimated molecular weight of 26,700 daltons.

Radioactive (^{51}Cr) sodium chromate is routinely used in clinical medicine to measure red cell survival (1). After transfusion of ^{51}Cr-labeled red blood cells, up to 15% of the ^{51}Cr may be eluted during the 24-hour posttransfusion period (2,3,4). The cause of this early elution has not been ascertained. Studies initiated in this laboratory to explore this phenomenon led to an unexpected finding which is reported in this work.

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Briefly, it has been demonstrated that ^{51}Cr binds to a site other than the hemoglobin monomeric subunits.

MATERIALS AND METHODS

Approximately 18 ml of blood was collected from a healthy volunteer into 2.7 ml of acid-citrate-dextrose.* The blood was then labeled with radioactive chromium** in a final concentration of 20 microcuries per ml of blood by incubating the chromium and the blood at 37°C for 30 minutes. The blood was diluted with an equal volume of 5 mM phosphate buffer in 0.15 M sodium chloride with a pH of 8.0 and centrifuged at 1500 X g for 10 minutes at 4°C. The red cells were washed with 40 ml of this same buffer by centrifugation at 1500 X g for 7 minutes on three separate occasions.

The red cells were lysed after the final wash by adding 1 ml of the packed cells to 39 ml of 5 mM phosphate buffer (pH 8.0) or else by freezing and thawing 1 ml of the packed cells and then diluting the hemolysate to 40 ml with the phosphate-sodium chloride buffer. In either case, the hemolysate was then centrifuged at 20,000 X g for 10 minutes at 4°C to remove the red cell membranes. The upper 5 ml of the supernatant was removed for use in this study. This supernatant was then prepared for electrophoresis by diluting an aliquot in the sodium dodecyl sulfate† solvent described by Fairbanks et al (5) and incubating for 2 hours at 37°C to reduce any disulfide bonds which might be present.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis†† was performed according to the method of Fairbanks et al (5). The gels were prepared and stained according to this method with the exception of the molecular weight determination when gels containing 10% acrylamide were used. Following staining, gels were photographed with Polaroid type 108 color film or type 105 positive/negative black and white film. The stained gels were quantitated by scanning in a spectrophotometer§ at 525 nm.

The molecular weight of chromium binding protein II was determined by SDS-PAGE. Four proteins of known molecular weight were used as markers for the molecular weight estimation. The proteins used in this study were ribonuclease A, MW 12,700 (6); chymotrypsinogen, MW 25,700 (7); aldolase, subunit MW 40,000 (7); and ovalbumin, MW 43,500 (6). The relative mobilities‡ were determined by the formula: $R_f = (X/T)(l_1/l_2)$, where X is the distance of the band from the origin, T is the distance of the tracking dye from the origin, l_1 is the length of the gel prior to staining, and l_2 is the length of the gel following staining. A computerized weighted regression analysis was performed to estimate the unknown molecular weight. This computer program operates on both BASIC and FORTRAN IV, and was available through the courtesy of Drs. D. Rodbard and A. Chrambach, National Institutes of Health, Bethesda, Maryland.

Commercially available hemoglobins were obtained from 4 sources: Calbiochem, La Jolla, Calif., #3747 Hemoglobin (Human), 2X cryst. A grade; Mann Research, New York, N. Y., #816 Hemoglobin (Human), 2X cryst. 100%; Sigma

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*ACD, National Institutes of Health, Formula A
**$Na_2{}^{51}CrO_4$, Squibb, Princeton, N. J.
†SDS
††SDS-PAGE
§Model 2520 Gel Scanner with Model 2400 Spectrophotometer, Gilford Instruments, Oberlin, Ohio
‡R_f

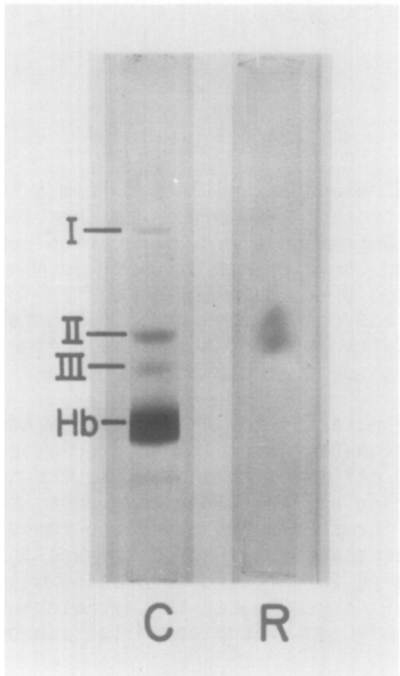

FIGURE 1

Homogeneity of protein II. Gel C is the electrophoretic pattern of the soluble protein fractions prepared from human erythrocytes on the day of collection. The three major non-hemoglobin proteins are indicated with Roman numerals and "Hb" indicates the location of the hemoglobin monomer. Gel R is a re-electrophoresis of band II. The location of band II was estimated on an unstained gel, that portion of the gel was removed and minced. This gel mince, along with additional SDS solvent, was layered onto a fresh gel and electrophoresed. Only one protein band appears after re-electrophoresis; this is in the location of band II. There is no protein located in the position of the hemoglobin monomer.

Chemical Co., St. Louis, Mo., #H7379 Hemoglobin, Type IV, Human, 2X cryst. The hemoglobins were dissolved in distilled water and electrophoresed to establish control values.

Both chromium-labeled and non-labeled samples were studied. Each sample was electrophoresed on two gels; one of these was stained in order to visualize the protein bands and the other was sliced into 1.2 mm sections and counted in a gamma counter for radioactivity.

RESULTS

Four major protein bands were visualized after staining (Figure 1, gel C); these bands appeared as optical density peaks when scanned in a spectrophotometer (Figure 2). The chromium label in these gels appeared as three peaks

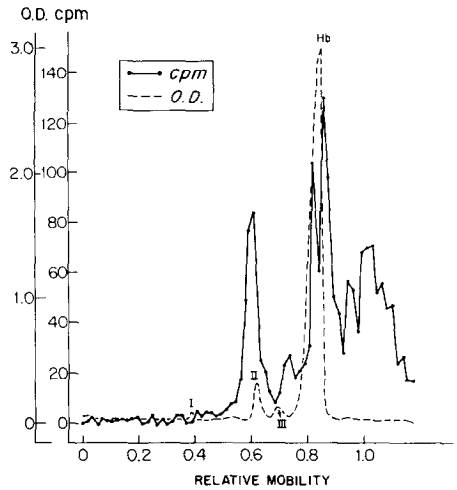

FIGURE 2

The ^{51}Cr radioactivity and optical density peaks of the SDS-electrophoresed protein. Large amounts of ^{51}Cr radioactivity are found to be coincident with the 525 nm optical density peaks of protein band II and the hemoglobin monomer. The third radioactivity peak is near the location of the tracking dye ($R_f \approx 1.0$). When chromium in a phosphate buffer is electrophoresed, its radioactivity peak is found to be associated with the tracking dye. Controls were run to determine if chromium was nonspecifically bound by the SDS denatured protein. The soluble fraction was first treated with the SDS solvent and then either hexavalent or trivalent chromium was added. In either case, only one radioactivity peak was observed; this was near the tracking dye, indicating that chromium did not bind to any proteins once denatured with SDS.

(Figure 2); the first of these radioactivity peaks was associated with the protein band II, the second peak with the hemoglobin monomer band, and the third with the location of the low molecular weight tracking dye. Protein bands I and III did not contain any appreciable amount of radioactivity. Of the estimated radioactivity layered onto the gels, a total of 70% to 90% was accounted for in the three major radioactivity peaks. The hemoglobin monomer accounted for 40% to 56% of this total, the low molecular weight components for 20% to 42%, and protein band II for 17% to 23%. Of the total optical density observed, 91% to 92% appeared as the hemoglobin monomer and 5% to 6% as protein band II.

Hemoglobin preparations obtained from commercial sources also had bands

other than the hemoglobin monomer. One of the bands in each preparation was observed in the same location as that of protein band II isolated from fresh hemoglobin.

The homogeneity of protein band II was established by re-electrophoresis of that portion of the unstained gel which corresponded to protein band II. The re-electrophoresed protein migrated as a single band and appeared in the location of protein band II (Figure 1, gel R). The radioactivity associated with the re-electrophoresed protein also moved as a single band and appeared in the location of the protein band II radioactivity peak.

A molecular weight of 26,720 daltons was computed for protein band II. The correlation coefficient was 0.998 which was significant at the 0.01 level.

DISCUSSION

It has been reported (8,9,10) that the majority of chromium associated with the red cell is attached to the hemoglobin molecule, specifically to the β-chain. Haut et al (11) have utilized starch gel electrophoresis to demonstrate the presence of non-hemoglobin proteins in the soluble fraction of red blood cells. Although the identity of protein II is unknown, it is not the hemoglobin monomeric subunit. The soluble protein fraction was dissolved in SDS containing dithiothreitol which dissociates proteins into subunits and prevents their reassociation. Furthermore, the estimated molecular weight of this protein was 26,700 which is lower than the reported value of 30,300 to 31,700 for a hemoglobin dimer (12). Finally, the re-electrophoresis of protein band II resulted in only a single band with no protein in the location of the hemoglobin monomer. If a monomer-dimer equilibrium existed, protein would have been observed in the location of the hemoglobin monomer. These data indicate that chromium was binding to a protein other than the hemoglobin monomer and that it is unlikely that this protein is the hemoglobin dimeric subunit.

Since protein band II represents an additional binding site for chromium within the red blood cell, its enzymatic and structural role warrants further investigation.

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REFERENCES

1. Ebaugh, F. G., Emerson, C. P., and Ross, J. F. (1953) J. Clin. Invest., 32, 1260-1276.

2. Mollison, P. L. (1961) Clin. Sci., 21, 21-36.

3. Mollison, P. L., and Veall, N. (1955) Brit. J. Haematol., 1, 62-74.

4. Szymanski, I. O., and Valeri, C. R. (1970) Brit. J. Haematol., 19, 397-409.

5. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochem., 10, 2606-2617.

6. Rodbard, D., and Chrambach, A. (1971) Analytical Biochem., 40, 95-134.

7. Weber, K., and Osborn, M. (1969) J. Biol. Chem., 244, 4406-4412.

8. Malcolm, D., Ranney, H. M., and Jacobs, A. S. (1963) Blood, 21, 8-20.

9. Pearson, H. A. (1963) Blood, 22, 218-230.

10. Pearson, H. A., and Vertrees, K. M. (1961) Nature (London), 189, 1019-1020.

11. Haut, A., Tudhope, G. R., Cartwright, G. E., and Wintrobe, M. M. (1962) J. Clin. Invest., 41, 579-587.

12. Harper, H. A. (1969) Review of Physiological Chemistry, p. 210, Lange Medical Publications, Los Altos.