MEMBRANE PROTEIN SYNTHESIS BY RABBIT RETICULOCYTES[+]

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Received April 5,1974

Summary: Intact rabbit reticulocytes synthesize several classes of membrane proteins ranging in molecular weight from 20,000 to 200,000. The synthesis is inhibited by cycloheximide, and treatment with ribonuclease does not significantly alter the gel pattern.

Introduction

In the differentiation of the mammalian red cell, the reticulocyte is the immediate precursor of the mature erythrocyte, and is the last stage at which protein synthesis still takes place (1,2). Although the overwhelming product made by the reticulocyte is hemoglobin, the synthesis of other proteins has been demonstrated (3-5). In addition, translation of exogenous messenger RNA by the cell-free lysate has been shown to occur (6-8).

Biosynthesis of reticulocyte membrane proteins was first shown by Harris and Johnson (9). Recently, Lodish, working with reticulocytes obtained from phenylhydrazine treated rabbits, reported the synthesis of two predominant species of membrane proteins by the intact cell, and by the stroma-free lysate (10). In this communication, we present evidence that several molecular weight classes of membrane proteins are synthesized by the intact rabbit reticulocyte.

Materials and Methods

Preparation of Reticulocyte-Rich Blood: Rabbits, 2-3 kg in weight, were bled from the ear vein for four successive days; 15-20 ml of blood

[+]This work was supported by grants #GM-16389, HD-07173 and HL-13979 from the National Institutes of Health.

per kg of body weight were collected at each bleeding. On the sixth day, blood was withdrawn by cardiac puncture. A reticulocyte count of 20-25% was usually obtained, as determined by methylene blue staining. The cells were centrifuged and washed three times with the isotonic salt solution (RS) described by Lingrel and Borsook (11), and the buffy coat was removed. No attempt was made to separate the reticulocytes from the erythrocytes.

Pre-Incubation of Cells: An incubation medium, modified from the reagent mixture described by Lingrel and Borsook (11), was prepared from the following: (a) A mixture of amino acids, each 1 mM in RS, 54.0 ml; (b) 0.25 M $MgCl_2$ + 10% glucose in RS, 2.7 ml; (c) TRIS-HCl, 0.164 M, pH 7.75, 27.0 ml; (d) trisodium citrate, 0.01 M in RS, 21.6 ml; (e) $NaHCO_3$, 0.01 M in RS, 32.4 ml. Rabbit serum, dialyzed against the above, was added to 10%.

2 ml of cells were incubated with 5.2 ml of the incubation medium. Ferrous ammonium sulfate was added to a final concentration of 1 mM, and isoleucine to 0.05 mM. 0.05 ml of a solution of penicillin (5000 units/ml) and streptomycin (5000 μg/ml) were added prior to incubation. The pH was adjusted to 7.5 and the cells incubated for 30 minutes at 37°C with gentle shaking. The final volume was 8 ml.

Washing of Cells: Following pre-incubation, the cells were centrifuged and washed with incubation medium minus isoleucine.

Incubation of Cells: The same protocol was followed as for the pre-incubation step, except that C^{14}-isoleucine was added to a final concentration of 5-10 μCi/ml of incubation solution (specific activity 313 mCi/mM, purchased from New England Nuclear). The addition of serum and the 30 minute pre-incubation period were found to significantly increase the incorporation of isotope into protein.

Preparation of Membranes and Analysis: Following the incubation, cells were washed twice with RS, and lysed by addition of ten volumes of hypotonic sodium phosphate buffer, as described by Dodge et al (12).

In some experiments, the lysate was incubated with pancreatic ribonuclease (Worthington), 10 μg/ml lysate, for 60 minutes at 37°C. Membranes were then washed with hypotonic buffer until most of the hemoglobin was removed.

Protein radioactivity was determined by precipitation with 5% trichloroacetic acid; the precipitates were collected on glass fibre filters and counted with Instagel (Packard Instruments). Hemoglobin was measured by absorbance at 576 mμ, using a molar extinction coefficient of 15.8×10^3 and by analysis with the benzidine reagent (13). Membrane protein was determined according to the procedure of Lowry et al (14).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate and the staining procedures were carried out as described by Fairbanks et al (15). For detection of radioactivity, gels were sliced with a razor blade device (Bio-Rad. Inc.), and gel slices incubated overnight at 37°C in a toluene cocktail containing 0.4% Omnifluor and 3% Protosol (New England Nuclear).

Results and Discussion

Fig. 1 shows the incorporation of C^{14}-isoleucine into hemoglobin

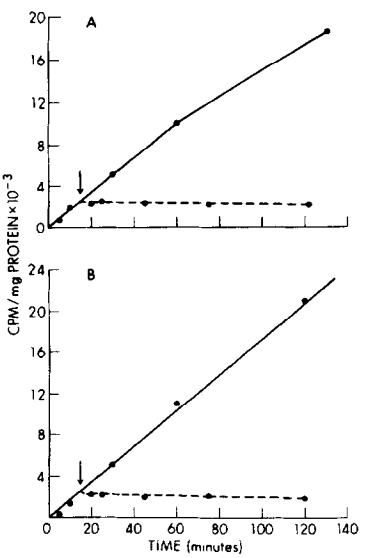

Figure 1. Incorporation of C^{14}-isoleucine into membrane proteins (A) and hemoglobin (B) by intact rabbit reticulocytes. Cycloheximide (0.9 mg/ml cell suspension) was added at 15 minutes (----).

and membrane protein over a 2 hour time period. Incorporation into membrane proteins is linear for 60 minutes and still increasing at 2 hours. In other experiments, incorporation of C^{14}-isoleucine into both hemoglobin and membrane protein was maintained up to 7 hours. Addition of cycloheximide completely inhibits further incorporation of radioactivity. No significant incorporation was noted with erythrocytes from untreated rabbits (reticulocyte count 1-2%) incubated in an identical manner. These results suggest that membrane proteins are synthesized by the reticulocyte.

The newly synthesized proteins were further analyzed by electrophoresis on polyacrylamide gels in sodium dodecyl sulfate. This is shown in Fig. 2. A parallel gel of membrane proteins stained with Coomassie

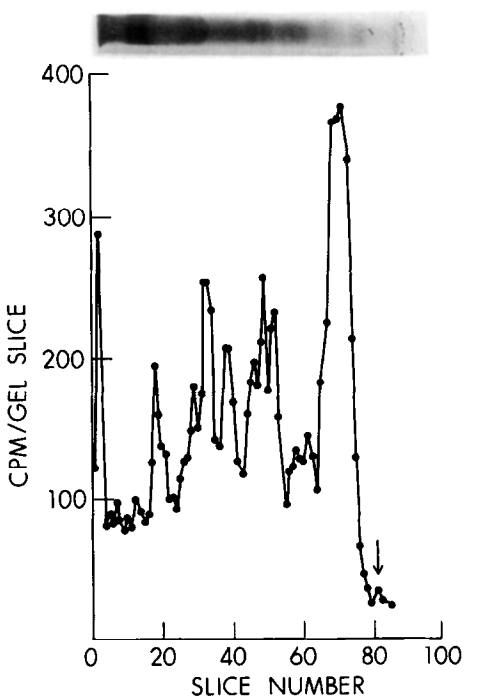

Figure 2. Distribution of C^{14} label in the reticulocyte membrane proteins after incubation of intact cells with C^{14}-isoleucine for 2 hours. Electrophoresis on 5.6% polyacrylamide gels in 1% sodium dodecyl sulfate. The gel at the top is a duplicate Coomassie stained gel. The large radioactive peak (slice number 65-75) close to the front of the gel contains hemoglobin.

blue is included in the figure. Relative migrations of radioactive peaks correspond in most cases with bands stained with Coomassie blue, as determined with help of a comparator on a photograph of the gel. No differences in radioactive peaks or Commassie blue-stained bands were seen in membranes treated with ribonuclease.

Our data suggest that several classes of membrane proteins, spanning a range of molecular weights from 20,000 to 200,000, are synthesized by the intact rabbit reticulocyte. The similarity in the pattern of radioactivity with and without ribonuclease suggests that peptides bound to ribosomes do not significantly contribute to the pattern observed, but the extent to which proteins of cytoplasmic membranes contribute to this pattern remains to be established.

We consistently observed a low content of radioactivity in the region of molecular weight 150,000, corresponding to gel slices 10-15. It appears that these proteins, which perhaps include spectrin (16), are synthesized to a lesser extent than the other classes of proteins.

The differences in our observations from those reported by Lodish (10) may be due to different procedures used in induction of reticulocytosis, or perhaps to the C^{14}-amino acid or incubation conditions employed. These questions are currently under investigation.

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