Nascent Globin Chains from Rabbit Reticulocyte Ribosomes

ACCUMULATION OF THE COMPLETED \( \alpha \) CHAIN

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SUMMARY

Evidence is presented to show that rabbit reticulocyte ribosomes contain a significant component of completed \( \alpha \)-globin which is still attached to tRNA (\( \alpha \)-globyl-tRNA). Additional data are presented to show that contamination by labeled supernatant hemoglobin or labeled \( \alpha \)-globin from the free \( \alpha \)-globin pool present in reticulocytes is not a significant factor in these results.

Some 4.6% of the nascent \( \alpha \)-globin chains are present as \( \alpha \)-globyl-tRNA, instead of 0.71% as predicted on the basis of the assumption that the size distribution of nascent globin chains is uniform. On the other hand \( \beta \)-globyl-tRNA comprises 0.69% of the nascent \( \beta \)-globin chains. This value coincides closely with the predicted value for nascent \( \beta \)-globin chains uniformly distributed in size along the polysome. Further evidence is presented to show that both \( \alpha \)-globyl-tRNA and \( \beta \)-globyl-tRNA exhibit the kinetic properties expected for normal intermediates of soluble hemoglobin biosynthesis following inhibition of the initiation of protein synthesis by pactamycin.

The availability of this methodology has made feasible analyses directed at the study of the globyl-tRNA molecules. The \( \alpha \) - and \( \beta \)-globin chains of rabbit hemoglobin each contain 3 tyrosine residues in their amino acid sequence (2). The COOH-terminal ends of \( \alpha \) - and \( \beta \)-globin molecules consist of the amino acid sequences -Lys-Tyr-Arg and -Lys-Tyr-His, respectively. Since the biosynthesis of hemoglobin is known to proceed from the NH\(_2\)-terminal end toward the COOH-terminal end (3) an analysis of the purified peptidyl-tRNA fraction for the presence of the COOH-terminal dipeptides tyrosyl arginine and tyrosyl histidine, following tryptic digestion, has permitted a determination of the amounts of \( \alpha \)-globyl-tRNA and \( \beta \)-globyl-tRNA in that fraction.

While \( \beta \)-globyl-tRNA exists to the extent predicted by a uniform distribution of sizes of nascent \( \beta \)-globin peptides the \( \alpha \)-globyl-tRNA was found to be present in an amount 6 times greater than the theoretical value predicted on the basis of the assumption of a uniform distribution of sizes of nascent \( \alpha \)-globin peptides.

Analyses of the \( \alpha \) - and \( \beta \)-peptidyl-tRNA obtained from uniformly labeled reticulocyte ribosomes following inhibition of further initiation with the antibiotic pactamycin indicates that the globyl-tRNA components of peptidyl-tRNA possess the kinetic properties expected for ribosomal intermediates in the biosynthesis of soluble hemoglobin.

EXPERIMENTAL PROCEDURE

Reagents—Cycloheximide, bovine hemin (twice crystallized), and ribonuclease D (five times crystallized, protease-free) from bovine pancreas were purchased from Sigma Chemical Co., St. Louis, Mo. Sarsomycin was generously donated by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone was obtained from Worthington Biochemical Corp., Freehold, N.J. Pactamycin was donated by the Upjohn Co., Kalamazoo, Mich. Penicillin G was purchased from Rutland Chemicals Co., Cleveland, O. Streptomycin sulfate, U.S.P., was acquired from General Biochemicals, Chagrin Falls, O. Diethylaminoethyl cellulose (DE-52) and carboxymethyl cellulose (CM-32) were purchased from Bio-Rad Laboratories, Richmond, Calif. Aquasol and Liquifluor were obtained from New...
England Nuclear, Boston, Mass. L-[3,5-3H]Tyrosine was purchased from Amersham-Searle Corp., Arlington Heights, Ill. (specific activities ranged from 33 to 40 Ci per mmole). L-[4C]-Tyrosine, 455 Ci per mole, was ordered from Schwarz-Mann, Orangeburg, N.Y. Nitrocellulose filters (0.4 μm pore size) were from Schleicher and Schuell Co., Keene, N.H. The synthetic dipeptides, L-tyrosyl-L-arginine and L-tyrosyl-L-histidine, were prepared by Cyclo Chemical, Los Angeles, Calif. All other reagents used were reagent grade.

Methods—Rabbit reticulocytes were obtained as described by Slabaugh and Morris (1). Hematocrits ranged from 12 to 16. The blood was passed through loose glass wool before the reticulocytes were washed twice at 4° in the 0.97% NaCl solution (RS) described by Lingrel and Borsook (4).

Standard Incubation Conditions—A suspension of reticulocytes was incubated in a modified medium of Lingrel and Borsook (4). Plasma from the same rabbit was dialyzed 1 hour against 35 volumes of cold RS prior to use in the incubation medium. The amino acid mixture of Lingrel and Borsook (4) was used except that hydroxyproline was omitted and L-asparagine was added to a final concentration of 0.55 mM in the incubation medium (5). Nonradioactive t-tyrosine was added to the isotopically labeled tyrosine to a final concentration of 0.021 mM in the incubation medium. This concentration of L-tyrosine was used for all incubations unless otherwise indicated. All incubations were performed at 37°. After an initial 2-min warm-up period the radioactive tyrosine was added to the reaction mixture. This addition of radioactivity defined zero time of incubation. The incubation was terminated by pouring the entire incubation mixture, or suitable aliquots thereof, into a 12-fold volume of ice-cold RS containing cycloheximide at a concentration of 16.5 μg per ml (0.059 mm). The cells were then collected by centrifugation and washed once more with fresh RS containing cycloheximide.

Preparation of Ribosomal Pellets—The washed reticulocytes were lysed for 10 min with 4 volumes of 2.5 mM magnesium chloride containing cycloheximide (0.69 mm) and 0.21 mM spermycin. The solution was then made isotonic by the addition of 1 volume of 1.5 M sucrose-0.15 M potassium chloride. Cell debris was removed by centrifugation at 20,000 X g for 30 min. The supernatant solution was then centrifuged at 64,000 X g for 3½ hours to obtain the radioactive ribosomal pellets. Where indicated, the ribosomal pellets were resuspended in Medium B (6) and resedimented by sedimentation as before to yield washed (two times) ribosomes. The concentration of ribonucleoprotein was determined by measuring the absorbance at 260 nm using an absorption coefficient of 11.3 for a concentration of 1 mg per ml (7).

Preparation of Peptidyl-tRNA—Ribosomal pellets were resuspended in a small volume (approximately 1 ml) of 0.25 M sucrose containing cycloheximide (0.059 mm) and 0.14 mM spermycin. The ribosomal suspension was then used to prepare peptidyl-tRNA according to the method of Slabaugh and Morris (1). It has been found that reduction of the urea concentration of Buffers I and II from 8.0 to 7.6 M avoids the occasional problem of crystallization of the urea solutions at 4°. This modification has been employed throughout these studies. The pooled fraction containing the purified peptidyl-tRNA was reduced to a volume of approximately 1.8 ml by ultrafiltration in an Amicon cell with a UM-2 Diaflo membrane. The concentrated sample was then dialyzed against three 1500-ml portions of deionized water, lyopholized, and stored at -21°.

Preparations of Globin Uniformly Labeled with [14C]Tyrosine—Washed reticulocytes were incubated in the presence of [4C]-tyrosine as described above, except that the tyrosine concentration in the medium was 0.1 mm. Penicillin and streptomycin were added to the reaction mixture to a final concentration of 0.11 mg per ml of each. Incubations were allowed to proceed at 37° for 3½ to 4 hours. The cells were washed, lyzed, and the postribosomal supernatant used to prepare hemoglobin according to the method of Winterhalter et al. (8). Hemoglobin concentration was determined by the method of Austin and Drabkin (9). Globin was prepared by the cold-acid-acetone method (10) and stored at -20° as a lyophilized powder.

Separation of Uniformly Labeled α- and β-Globin Chains—The α- and β-globin chains of 4C-labeled rabbit globin were separated on carboxymethyl cellulose (CM-32) columns (1 x 22 cm) with a nonlinear gradient modified from the procedure of Rabindrovitz et al. (11). The gradient was generated by placing concentration multiples of 1-, 3-, 5-, 7-, 1-, 7-, and 9-fold of the starting buffer (0.2 M formic acid-0.02 M pyridine) in successive chambers of a 10-chamber rectangular Varigrad (Buehler Instruments Inc., Fort Lee, N.J.). The contents of each chamber (50 ml) were 0.05 M in β-mercaptoethanol (12). Prior to chromatographic separation the globin samples were dialyzed overnight against 0.05 M β-mercaptoethanol and then adjusted to 0.2 M formic acid, 0.02 M pyridine. Globin (45 mg or less) was applied to the column and eluted at a flow rate of 14 to 16 ml per hour. The separated α- and β-globin chains were then lyophilized and each was rechromatographed on a CM-32 column by the same procedure in order to obtain further purification. Lyophilized samples of separated globin chains were stored at -20°. The purity of the separated α- and β-globin chains obtained in this manner was established by the addition of nonradioactive carrier globin and digestion of the mixture with trypsin at 37° for 4 hours as described below. The six tryptophan-containing peptides (αT14, αT16, αT116, βT14, βT114, βT16) were separated according to the method of Hunt et al. (13) and analyzed for radioactivity. By this means it could be shown that the α chain preparation contained approximately 0.82% β chain while the β chain preparation contained approximately 1% contamination by α-globin.

Analysis of Naseent Globin Chains—The lyophilized sample of labeled peptidyl-tRNA was resuspended in 1.0 ml of water containing 0.1 mg of pancreatic RNase, incubated at 37° for 25 min, and then lyophilized. After redissolving in 0.15 ml of 0.1 N NaOH the material was incubated for 3½ hours at 37° in order to cleave the peptidyl-tRNA ester bond. The solution was then neutralized with 1 N HCl to a pH of 5.4 to 5.6 as determined with pH indicator paper. Purified rabbit α- and β-globin chains of known radioactivity content (uniformly labeled with [14C]tyrosine) were then added as an internal standard (3). Nonradioactive globin was added, if necessary, to give a mass of 3 to 4 mg of protein in the sample. The synthetic dipeptides, L-tyrosyl-L-arginine (αT15) and L-tyrosyl-L-histidine (βT16), were added as carrier peptides (50 nmoles each) prior to trypsic digestion.

Tryptic Digestion—Tryptic digestion was carried out in 0.1% NaHCO3 (14) at a final globin concentration of 3 to 4 mg per ml. Tryptsin was added in an amount equal to 2% (w/w) of the total globin present. After 2 hours incubation at 37°, 1% (w/w) trypsin was again added and the incubation continued for an additional 2 hours. Samples were then frozen and lyophilized.

Separation of Peptides—Separation of the tyrosine-containing peptides from rabbit globin was performed by the two-dimensional method of Hunt et al. (13). Electrophoresis was con-
duced in a Gilson model D high voltage electrophorator. Tryptic peptides have been numbered according to their position of occurrence relative to the NH\textsubscript{2}-terminal end of the \(\alpha\) and \(\beta\)-globin chains of rabbit hemoglobin (15). The appropriate areas containing the radioactive tyrosine peptides were removed from the electrophoretogram, remaining solvents removed in vacuo, and the paper was then cut into small sectors and placed in scintillation vials for the elution procedure. Three extractions with 2 ml of 0.01 N HCl were performed at 80°. The eluates were pooled into scintillation vials and lyophilized.

Counting of Radioactivity—The tryptic peptides were dissolved in 0.01 N HCl and combined with 10 ml of Aquasol and counted in a Packard liquid scintillation spectrometer model 3310. Counting efficiencies were determined by the channels ratio method for doubly labeled samples. Counting efficiencies of samples containing a single radioisotope were established by internal standardization with \(^3\)H- or \(^4\)C-labeled toluene of known radioactivity content (New England Nuclear, Boston, Mass.). All data are expressed as decompositions per min (dpm) as determined from the observed counts per min and the counting efficiency.

The elution of radioactive materials during column chromatography was monitored by placing 25- to 50-ml aliquots of the eluate fractions in 0.5 ml of H\textsubscript{2}O, 5 ml of Aquasol, and counting in a Nuclear Chicago, Unilux 1, liquid scintillation counter.

Least squares calculations were achieved with the CDC 6500 computer of Michigan State University.

RESULTS

Purified Peptidyl-tRNA Is Free of Contamination with Soluble Hemoglobin—The analyses conducted in this study require that purified peptidyl-tRNA be free of significant amounts of contamination by soluble (labeled) hemoglobin. The two analyses described below were performed to assess this degree of contamination.

A mixture of nonradioactive reticulocyte ribosomes and purified \(^3\)H-labeled hemoglobin was prepared (see legend of Table I). This mixture was then subjected to the procedure for preparation of peptidyl-tRNA (1). Radioactivity present in the purified peptidyl-tRNA fraction thus represents the extent of contamination by hemoglobin in that fraction. Results from the two separate analyses appear in Table I. These results indicate that not more than 0.030% of the labeled hemoglobin originally added remains in the purified peptidyl-tRNA fraction.

Labeling of Ribosomes in Whole Reticulocyte—Fig. 1 shows the time course of incorporation of \(^3\)H-tyrosine into the ribosomes and into soluble hemoglobin of intact reticulocytes. The incorporation of radioactivity into ribosomes reached a constant value by 4 min after addition of \(^3\)H-tyrosine to the incubation medium. The specific radioactivity of the ribosomes remained constant for at least the next 16 min. The incorporation of radioactivity into soluble hemoglobin was linear for at least the first 20 min of incorporation. These results are consistent with those obtained by Hunt et al. (13). The constant level of radioactivity found in the ribosomal fraction after 4 min of incubation assures that a steady state of labeling of precursor pools and nascent protein has taken place. Nascent globin chains prepared from cells collected at 10 min of incubation thus possess uniform specific activity of the 6 tyrosine residues present in the nascent globin chains.

Analysis of \(^3\)H-Tyrosine-labeled Peptidyl-tRNA—Rabbit reticulocytes were incubated in a medium containing \(^3\)H-tyrosine for 10 min at 37°. The ribosomal pellets obtained from the labeled reticulocytes were used to prepare the purified peptidyl-tRNA fraction. Following the addition of \(^4\)C-labeled \(\alpha\) and \(\beta\)-globin chains to the peptidyl-tRNA as internal standards, the mixture was digested with trypsin and the tyrosine-containing tryptic peptides were isolated and analyzed as described under "Experimental Procedure."

![Fig. 1. Time course of incorporation of \(^3\)H-tyrosine into ribosomes and into soluble hemoglobin of rabbit reticulocytes.](http://www.jbc.org/content/170/2/7440/F1)

Add 3H-hemoglobin found in purified peptidyl-tRNA fraction

Rabbit reticulocytes (0.5 ml packed cell volume) were incubated as described under "Experimental Procedure." The tyrosine concentration in the incubation medium was 0.1 mM. Tritium-labeled alanine, valine, and leucine (0.5 mCi each) were added and the incubation was allowed to proceed for 45 min at 37°. The post-ribosomal supernatant fraction was dialyzed against 0.1 M sodium acetate (pH 5.6) and passed through a column of CM-cellulose chromatography (see "Experimental Procedure"). This mixture was then subjected to the procedure for preparation of peptidyl-tRNA (1). Radioactivity present in the purified peptidyl-tRNA fraction thus represents the extent of contamination by hemoglobin in that fraction. Results from the two separate analyses appear in Table I. These results indicate that not more than 0.030% of the labeled hemoglobin originally added remains in the purified peptidyl-tRNA fraction.

| Experiment | \(^3\)H-Hemoglobin added | Unlabeled ribosomes added | \(^3\)H-Hemoglobin recovered in purified peptidyl-tRNA fraction |
|------------|--------------------------|---------------------------|------------------------------------------------------------|
| I          | dpm \(\times 10^{-4}\)    | mg                        | dpm %                                                      |
| I          | 25.8                     | 40.0                      | 7,850 0.090                                                |
| II         | 18.1                     | 48.4                      | 4,450 0.025                                                |

| RIBOSOMAL BOUND RADIOACTIVITY \(\times 10^{-4}\) (CPM/DO) | MINUTES | SOLUBLE RADIOACTIVE PROTEIN \(\times 10^{-1}\) (CPM/DO) |
|-------------------------------------------------------------|---------|----------------------------------------------------------|
| 0                                                           | 10      | 20                                                       |

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\(\text{Fig. 1. Time course of incorporation of } \text{[H]}\text{-tyrosine into ribosomes and into soluble hemoglobin of rabbit reticulocytes.}
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The relative specific activities \(\left[\frac{3H}{^{14}C}\right] \) of the tryptic peptides are shown in Fig. 2. The \(\left[\frac{3H}{^{14}C}\right] \) intercepts were calculated by the method of least squares in order to obtain a relative measurement of the number of \(\alpha\) and \(\beta\) nascent chains present. The \(\left[\frac{3H}{^{14}C}\right] \) ratios observed in the COOH-terminal tryptic peptides (\(\alpha T15\) and \(\beta T16\) were used as a relative measurement of the number of completed \(\alpha\)- and \(\beta\)-globin chains in the peptidyl-tRNA fractions since only the completed globin chains in the population of nascent chains can yield those tryptic peptides upon hydrolysis (3).

The results of three independent experiments are shown in Table II. Each set of experimental data was analyzed as shown in Fig. 2 for Experiment I. It is apparent from these data that rabbit reticulocytes contain a significant component of \(\alpha\)-globin which is still attached to tRNA (\(\alpha\)-globyl-tRNA). Some 4.6% of nascent \(\alpha\)-globin chains are present as \(\alpha\)-globyl-tRNA. On the other hand, completed \(\beta\) chains attached to tRNA (\(\beta\)-globyl-tRNA) constitute only 0.7% of the nascent \(\beta\)-globin chains.

**Effect of Hemin**—The presence of a pool of free soluble \(\alpha\)-globin chains has been shown to be present in the reticulocyte (17, 18). It has also been reported that this pool is decreased in size if the reticulocytes are incubated with hemin (17). In order to examine the possible effects of hemin on the accumulation of \(\alpha\)-globyl-tRNA on the ribosomes two parallel incubations of rabbit reticulocytes were performed. One incubation mixture was conducted in the usual manner (see legend of Table II) while to the other was added hemin to a concentration of \(1 \times 10^{-4} \text{ M}\). Analysis of the six tyrosine-containing peptides from each preparation, conducted as before, revealed that hemin addition to the incubation medium did not alter the proportion of nascent \(\alpha\) or \(\beta\)-globin chains present as \(\alpha\)-globyl-tRNA or \(\beta\)-globyl-tRNA, respectively, in the purified peptidyl-tRNA fraction.

**Pactamycin-induced Decay of Radioactivity in Nascent Globin Chains**—The antibiotic pactamycin, at a concentration of \(10^{-5} \text{ M}\), has been shown to inhibit preferentially the initiation of protein synthesis in the reticulocyte (19, 20). Since elongation and release of nascent globin chains are not inhibited, preparations of purified peptidyl-tRNA obtained from reticulocytes that have been exposed to pactamycin should show a progressive decrease of radioactivity in the peptidyl-tRNA fraction with an increased time of incubation.

The effects of pactamycin addition to rabbit reticulocytes

![Fig. 2. Relative specific activities of the nascent globin peptides from purified peptidyl-tRNA. The ordinate represents the \(\left[\frac{3H}{^{14}C}\right] \) ratios obtained in Experiment I of Table II. Each tryptic peptide is positioned on the abscissa according to the position of the COOH-terminal amino acid of that tyrosine-containing tryptic peptide in the sequence of rabbit hemoglobin. Tryptic peptides have been numbered according to their position of occurrence relative to the NIH-terminal end of the corresponding rabbit globin chains. Lines drawn through each set of points thus represent the relative specific activities to be expected for each amino acid present in a uniform distribution of nascent chains on the polysome. The \(\left[\frac{3H}{^{14}C}\right] \) intercept has been used as a measure of the total nascent chains present and the ordinate value corresponding to \(\alpha T15\) or \(\beta T16\) has been used as a measure of \(\alpha\)-globyl-tRNA or \(\beta\)-globyl-tRNA present, respectively.

### Table II

**Analysis of \([\text{H}]\)tyrosine-labeled tryptic peptides from purified peptidyl-tRNA**

Rabbit reticulocytes (10 ml packed cell volume) were incubated for 10 min at 37°. The reaction mixture contained 2 mCi of \(\left[\text{H}\right]\)tyrosine (2421 pCi per pmole). The incubation conditions, preparation of peptidyl-tRNA, trypsin digestion and analysis of labeled tryptic peptides are described in detail under "Experimental Procedure." For each of the analyses 47,400 dpm of \(\left[^{14}\text{C}\right]\)tyrosine-labeled \(\alpha\)-globin and 50,100 dpm of \(\left[^{14}\text{C}\right]\)tyrosine-labeled \(\beta\)-globin were added as a uniformly labeled internal standard.

| Experiment | \(\left[\frac{3H}{^{14}C}\right] \) | \(\alpha\)-Globin peptide | \(\beta\)-Globin peptide |
|------------|----------------|----------------|----------------|
|            | \(T4\) | \(T5\) | \(T15\) | \(\alpha T15\) intercept | % | \(T4\) | \(T14\) | \(T16\) | \(\beta T15\) intercept | % | \(\alpha\)-Globyl-tRNA | \(\beta\)-Globyl-tRNA |
| \(\text{H}^{14}\text{C}\) | 1490 | 1130 | 84.9 | 118 | 4.56 | 89.1 | 14.7 | 0.88 | 122 | 0.72 | 6.1 |
| \(\text{H}^1\) | 94.4 | 71.7 | 5.38 | % | | 1410 | 232 | 13.9 | | | |
| \(\text{H}^{14}\text{C}\) | 127 | 97.8 | 6.93 | 160 | 4.33 | 112 | 22.6 | 1.04 | 154 | 0.68 | 6.7 |
| \(\text{H}^1\) | 86.4 | 64.6 | 5.31 | 107 | 4.96 | 88.3 | 15.6 | 0.82 | 118 | 0.69 | 6.5 |
| \(\text{H}^{14}\text{C}\) | 1300 | 1020 | 83.9 | 4.62 | 6.4 |
| \(\text{H}^1\) | | | | | | | | | |

* The \(\left[\frac{3H}{^{14}C}\right] \) intercepts were calculated by the method of least squares as described under "Experimental Procedure."

* Observed \(\left[\frac{3H}{^{14}C}\right] \) (of \(\alpha T15\) or \(\beta T16\), respectively) \(\times 100 \)/calculated \(\left[\frac{3H}{^{14}C}\right] \) intercept (of \(\alpha\)-peptides or \(\beta\)-peptides, respectively).

* Tritium content of the tryptic peptides is expressed as total decompositions per min \(\times 10^{-3}\) by equating \(\text{H}^1\) recoveries to observed \(\text{H}^4\) recoveries.
whose ribosomes were in a steady state of labeling (10 min at 37') is shown in Fig. 3. The specific activity of hemoglobin in the soluble phase of the reticulocyte was found to increase very little following pactamycin addition to the incubation medium, hence the amount of contamination of the ribosomal pellets with \(^{3}H\)-labeled hemoglobin should be similar in each of the preparations obtained at the respective time periods after pactamycin addition. However, the radioactivity found in the purified peptidyl-tRNA fraction prepared from samples withdrawn following incubation in the presence of pactamycin decrease rapidly with time. After 5 min of incubation in the presence of pactamycin, only 8.9% of the original radioactivity remained associated with peptidyl-tRNA.

The radioactivity content of each of the six tyrosine-containing tryptic peptides in each of the four samples was analyzed. These data are presented in Fig. 4. The radioactivity present in all tyrosine-containing peptides (including \(\alpha T15\) and \(\beta T16\)) declines precipitously after pactamycin addition. Peptides \(\alpha T4\) and \(\beta T4\), closest to the NH\(_2\)-terminal portion of the respective globin chains, decline most rapidly following the addition of pactamycin. These results are consistent with completion and release of nascent peptides without initiation of new peptide chains.

Fig. 5 presents the relative radioactivity content of tryptic peptides near the NH\(_2\)-terminal portion (\(\alpha T4\), \(\beta T4\)) of the nascent peptides as compared to the radioactivity present in the COOH-terminal tryptic peptides (\(\alpha T15\), \(\beta T16\)). The proportions of \(\alpha\)-globyl-tRNA and \(\beta\)-globyl-tRNA in the nascent protein fraction were markedly increased with time after pactamycin addition. Since \(\alpha T15\) is derived only from globyl-tRNA while \(\alpha T4\) is derived from both globyl-tRNA and the other tyrosine-containing \(\alpha\)-globyl nascent peptides as well, one can determine that by 5 min after pactamycin addition approximately 24% of the nascent \(\alpha\)-globin peptides are \(\alpha\)-globyl-tRNA.

A similar calculation indicates that \(\beta\)-globyl-tRNA reaches approximately 7% of the nascent \(\beta\)-globin peptides by 5 min after pactamycin addition. The total radioactivity content of all components decreased rapidly following pactamycin addition, however. Collectively these data are consistent with the concept that the globyl-tRNA molecules are normal intermediates of soluble hemoglobin biosynthesis.

**DISCUSSION**

Evidence presented in this paper indicates the existence of a heretofore undetected accumulation of \(\alpha\)-globyl-tRNA on reticulocytes labeled in the steady state. Rabbit reticulocytes (10 ml packed cell volume) were incubated as described under "Experimental Procedure." At zero time 5 mCi of \(^{3}H\)tyrosine (6053 Ci per pmole) were added. After 10 min of incubation the first aliquot (10 ml) was withdrawn to serve as a control prior to pactamycin addition. After an additional 15 s, as indicated by the arrow, the incubation mixture was made 1 X 10\(^{-6}\) M in pactamycin by the addition of 0.6 ml of 0.5 X 10\(^{-4}\) M pactamycin in 0.9% NaCl solution (RS). Three further 10-ml aliquots were withdrawn at the time intervals indicated in the figure. Peptidyl-tRNA was prepared from each aliquot and the total radioactivity content was determined as described under "Experimental Procedures." Hemoglobin specific activities were measured in the postribosomal supernatant fractions.
bit reticulocyte polysomes. In the case of β-globyl-tRNA there is no such accumulation. If one were to assume that the size distribution of nascent globin chains is uniform (21, 22), then the α-globyl-tRNA might be expected to be 1/4 or 0.71% of the total nascent chains. In contrast to this prediction, the percentage of nascent α-peptides present as α-globyl-tRNA was found to be more than 6 times higher than predicted. Similar calculations for the nascent β chains would predict 1/4 or 0.69% β-globyl-tRNA in the nascent β-peptide fraction. In this case the analytical results coincide closely with the predicted value for the nascent β-peptides. A 6.4-fold excess of α-globyl-tRNA to β-globyl-tRNA exists in the peptidyl-tRNA fraction (Table II). In spite of the high ratio of α-globyl-tRNA to β-globyl-tRNA the over-all ratio of total nascent β chains to total nascent α chains, as determined from the intercepts of Fig. 2, was found to be 1/0.4, in close agreement with the finding reported by others (12, 21, 22). Various authors have, in the past, measured the percentage of nascent globin chains in the rabbit reticulocyte polysome corresponding to completed α-globin chains or β globin chains. However, these experiments were carried out using preparations which contained varying levels of contaminating soluble hemoglobin. This has led to a wide range of often conflicting results. Lippis et al. found no evidence of accumulation of completed α or β chains on the polysome (23). Hunt et al. found equal numbers of completed α and β chains attached to the polysome, with results varying from experiment to experiment. The latter authors reported the presence of from one complete globin chain per seven nascent globin chains to one completed globin chain per 60 nascent globin chains (21). Colombo and Bagioni found an excess of α-globin chains in a reticulocyte preparation (24). On the basis of the time required for the termination and release of globin chains Lodish and Jacobsen have predicted that 5% of the nascent globin chains on the reticulocyte ribosome should be completed α- and β-globin chains, both chains being present in equal proportions (25).

The present paper presents evidence to show that the amount of free hemoglobin contaminating the preparations of peptidyl-tRNA used to obtain the results reported here does not contribute significantly to the observed results. This contamination amounts to not more than 0.030% of the labeled hemoglobin present in the original ribosomal pellet. During the preparation of peptidyl tRNA from radioactively labeled reticulocytes, such as the preparations used to obtain the data in Table II, the average radioactivity due to soluble hemoglobin contamination of the unfractonated ribosomal pellet amounted to 6.1 x 10^6 dpm. This amount of radioactivity in the hemoglobin contaminating the ribosomal pellet will leave not more than 1800 dpm of contaminating hemoglobin in the purified peptidyl-tRNA fraction. Since the supernatant hemoglobin would be expected to be nearly uniformly labeled (3), and since tryptic digestion of globin produces six tyrosine-containing peptides, the radioactivity due to each contaminant tryptic peptide would not be expected to exceed 300 dpm. In the analyses reported in Fig. 2, tryptic peptide βT16 contained an average of 14,500 dpm. The total radioactivity in all the other tryptic peptides is even greater by at least 7-fold or more. Contamination from soluble hemoglobin (300 dpm) would introduce an uncertainty of about 0.014% to the reported value of 0.70% for β-globyl-tRNA (Table II). Similar considerations would introduce an uncertainty due to soluble hemoglobin contamination of approximately 0.015% to the reported value of 4.62% for α-globyl-tRNA (Table II). The uncertainty due to contamination of the other tryptic peptides is negligible. The results obtained with pactamycin reinforce this conclusion. Figs. 4 and 5 show that the preparations of purified peptidyl-tRNA contain true intermediates of globin biosynthesis. In particular, Fig. 5 shows quite clearly the effect of inhibiting initiation of new nascent chains. While there is a decrease in the amount of nascent protein attached to the ribosomes the effect is most pronounced among those nascent protein chains in the early stages of synthesis. Hence, the proportion of globyl-tRNA in the nascent protein fraction is increased markedly even though the absolute quantity of globyl-tRNA is reduced. Contamination of the peptidyl-tRNA fraction by soluble labeled hemoglobin would have obscured these changes.

A consideration of the ratio of αT15:βT16 found following pactamycin addition to the reaction mixture reveals an average αT15:βT16 ratio of 5.8 during the first 30 s after addition of the antibiotic. By 2.2 min in the presence of pactamycin that ratio has been reduced to 4.9 and reaches 2.3 after 5 min of incubation. These results are incompatible with contamination of the peptidyl-tRNA fraction with free α-globin chains which have been reported to be present in the reticulocyte (18).

Accumulation of α-globyl-tRNA to the extent of 4.6% of the total number of nascent α-peptides present indicates that one ribosome in 23 of those which are actively engaged in α-globin synthesis possess an α-globyl-tRNA. From the number of ribosomes per polysome which are synthesizing α-globin in the rabbit reticulocyte (22) one can estimate that approximately one of these polysomes in five contains an α-globyl-tRNA. Since α-globyl-tRNA would be expected to be the normal substrate for the release steps in α-globin biosynthesis the observed accumulation may be a reflection of a limitation of the rate of release of completed α-globin chains from the biosynthetic template.

The biological significance of the accumulation of α-globyl-tRNA on rabbit reticulocyte ribosomes and its relationship to the termination and release of both α and β globin chains in the biosynthesis of rabbit hemoglobin are currently under study in this laboratory.

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