Delayed Removal of N-Terminal Methionine from Nascent Globin Chains in Sickle-Cell Anemia Reticulocytes

(hemoglobin synthesis/initiation/peptide synthesis/peptide chromatography/polypeptide folding)

HARUO SUZUKI* AND HARVEY A. ITANO†

Department of Pathology, University of California, San Diego, La Jolla, Calif. 92037

Communicated by S. J. Singer, April 23, 1973

ABSTRACT Synthetic αT1 and βT1, the N-terminal tryptic peptides of α-chain and β-chain of hemoglobin, and MetaT1 and MetβT1, peptides in which N-terminal methionyl residues are peptide-bonded to αT1 and βT1, were prepared by the solid-state method of Merrifield. These synthetic peptides were used to establish conditions for chromatographic purification and analysis. When tryptic digests of nascent globin chains from rabbit and sickle-cell anemia reticulocytes incubated with 35S- and 3H-labeled amino acids were analyzed, radioactivity not present in tryptic digests of labeled hemoglobin appeared at the elution positions of MetaT1 and MetβT1. The fraction of nascent chains with N-terminal methionine was higher in sickle-cell anemia reticulocytes than in rabbit reticulocytes. If rate of peptide-chain elongation in polysomes is uniform, nascent human chains must attain a greater length before removal of the initial methionyl residue. Length of nascent chain at time of removal was calculated from two independent sets of data, one obtained from 35Smethionine incorporation into MetaT1, MetβT1, αT5, and βT5, and the other obtained from [3H]lysine and [3H]valine incorporation into MetaT1 and βT1.

Synthesis of globin chains in rabbit reticulocytes is initiated with a methionyl residue (1–7); this residue is removed during peptide-chain elongation, and valine is the N-terminal residue of these chains in rabbit hemoglobin. Wilson and Dintzis (1) found that about 15% of nascent α-chains in rabbit reticulocytes have N-terminal methionine, and Jackson and Hunter (4) estimated that the N-terminal methionine of nascent chains of rabbit α- and β-chains are removed when these chains are 15–20 aminoacid residues long. Yoshida and his coworkers (5) showed that nascent rabbit globin chains composed of more than 30 aminoacid residues have unblocked valine at the N-terminal position, whereas chains of less than 16 amino acids have N-terminal methionine and a small fraction of formyl methionine.

Presence of methionine at the N-terminus of short nascent chains of globin leads to low yields of the N-terminal valyl tryptic peptides, αT1 of α-chain and βT1 of β-chain, in relation to the other tryptic peptides of these nascent chains. Low yields are indeed evident in previous studies with rabbit reticulocytes; however, the decreases in yields appear to be at most 10–15% below expected values (8, 9). On the other hand, Kazazian and Itano (10) observed a decrease in yield of more than 50% in tryptic digests of nascent chains from sickle-cell anemia reticulocytes.

The basis for the differences in yields of the N-terminal tryptic peptides in rabbit and sickle-cell anemia reticulocytes was investigated. Chemically synthesized peptides with the aminoacid sequences of αT1 and βT1 and with a methionyl residue attached by a peptide bond to the N-terminus of each of these sequences were used to determine conditions for identification and chromatographic separation of these peptides in tryptic digests of nascent chains.

MATERIALS AND METHODS

Chemicals. tert-Butyloxy carbonyl amino acids used for peptide synthesis were purchased from Fox Chemical Co. Chloromethyl resin was a gift from Dr. J. Scottcher. Other chemicals used for peptide synthesis were reagent grade. L-[4,5-3H]lysine, 41.6 Ci/mmol, was purchased from Schwarz/Mann. L-[3H]valine, 1.11 Ci/mmol, and L-[35S]methionine, 75.8 Ci/mmol, were purchased from New England Nuclear Corp. L-[3H]Methionine, 38 Ci/mmol, was purchased from Amersham/Searle.

Peptide Analysis. Peptides were purified by automatic chromatography (11) on Aminex A5 and AG50W-X2 from Bio-Rad Laboratory. Peptides were eluted from the column at 50° with a linear pH gradient obtained by mixing 250 ml each of pyridine-acetic acid buffers of pH 3.1 and 5.0. In some analyses, peptides were eluted with 60–75 ml of pyridine-acetic acid buffer of pH 2.5 before the linear pH gradient was started. The radioactivity of each fraction was counted in a Beckman LS-232 liquid scintillation counter.

Peptide Synthesis. The following peptides were synthesized by the Merrifield method (12). α-Chain (rabbit, human)-αT1, Val-Leu-Ser-Pro-Ala-Asp-Lys; MetaT1, Met-Val-Leu-Ser-Pro-Ala-Asp-Lys. β-Chain-Rabbit: βT1(R), Val-His-Leu-Ser-Glu-Glu-Lys; MetaβT1(R), Met-Val-His-Leu-Ser-Glu-Glu-Lys. Human hemoglobin S: βT1(S), Val-His-Leu-Thr-Pro-Val-Glu-Lys; MetaβT1(S), Met-Val-His-Leu-Thr-Pro-Val-Glu-Lys. Yields of crude peptides were 70%. Peptides were purified by Bio-Rad P-2 gel filtration followed by Aminex A5 chromatography. Aminoacid analyses of peptides were performed on the Beckman aminoacid analyzer, model 116. The compositions of the synthesized peptides agreed with their respective sequences.

Reticulocyte Experiments. Labeling experiments of sickle-cell and rabbit reticulocytes with radioactive amino acids were done as described (13) with minor modifications. Puri-
Separation of Peptides. Column chromatographic separation of MetaT1 from αT1, MetβT1(R) from βT1(R), and MetβT1(S) from βT1(S) were performed on an Aminex A5 column with a linear pH gradient (Fig. 1). Each pair of these peptides eluted together when chromatographed with the same buffer on an AG50W-X2 column. MetaT1 and αT1 eluted at fraction 13 (39 ml) from a 0.9 × 13.0-cm column. MetβT1(R) and βT1(R) eluted at fraction 47 (141 ml) and MetβT1(S) and βT1(S) eluted at fraction 46 (138 ml) from a 0.9 × 19.5 cm column.

Incorporation of [35S]Methionine into Nascent Chains of Rabbit and Sickle-Cell Anemia Reticulocytes. Rabbit reticulocytes (10 ml) were incubated with 200 μCi of [35S]methionine (75.8 μCi/mmol) for 10 min at 37°, and the polynuclear and postribosomal fractions were prepared. Tryptic digests of these fractions were analyzed on Aminex A5 and AG50W-X2 columns. The results are shown in Fig. 2.

Tryptic digests of nascent chains showed radioactivity at the positions of methionine-containing peptides βT5, αT5, and MetβT1(R) from the Aminex A5 column (Fig. 2A). However, no radioactivity was observed at the position of peptide MetβT1(R) when the tryptic digest of the postribosomal supernatant were analyzed (Fig. 2B). Fractions 11–28 from Aminex A5 chromatography were collected for chroma-

RESULTS

Fig. 1. Separation of chemically synthesized peptides. A mixture of peptides, MetaT1 and αT1 or MetβT1 and βT1, was applied on an Aminex A5 column (0.9 × 19.2 cm), and eluted at 50° with a linear pH gradient of pyridine-acetic acid buffers from pH 3.1–5.0, 250 ml of each. The effluent stream was divided. One of the resulting streams was reacted with ninhydrin, after which transmittance at 570 nm was continuously recorded. The other stream was directed to a fraction collector. (A) 200 nmol of αT1 and MetaT1 were applied. The first peak was identified as MetaT1 and the second as αT1. (B) 200 nmol of βT1(R) and MetβT1(R) were applied. The first peak was identified as MetβT1(R) and the second as βT1(R). (C) 200 nmol of βT1(S) and 60 nmol of MetβT1(S) were applied. The first peak was identified as MetβT1(S) and the second as βT1(S).

Fig. 2. Incorporation of [35S]methionine into rabbit hemoglobin. Rabbit reticulocytes were incubated with [35S]methionine, 75.8 Ci/mmol, and the polynuclear fraction was obtained. One portion of the polynuclear fraction was digested with bovine RNase and then with trypsin. Globin was prepared from the postribosomal supernatants, and this globin was digested with trypsin. (A) A tryptic digest of nascent chains was loaded on a column of Aminex A5 resin and eluted with the same linear pH gradient as in Fig. 1. From left to right, the arrows indicate peptides βT5, αT5, and MetβT1(R). (B) A tryptic digest of the postribosomal supernatant was chromatographed as in (A). Arrows indicate the same peptides as in (A). (C) Fractions 12–28 from Aminex A5 chromatography (A in this Fig.) were collected and loaded on a column of AG50W-X2 (0.9 × 12.0 cm). From left to right, the arrows indicate MetaT1, αT5, and βT5. (D) Fractions 11–27 from the Aminex A5 chromatography (B in this Fig.) were collected and run on a column of AG50W-X2 (0.9 × 12.0 cm). Arrows indicate the same peptides as in (C).
tography on a different column (Fig. 2C and D). Radioactivity was observed at the position of MetαT1 when these fractions from nascent chain digests were analyzed on the AG50W-X2 column (Fig. 2C), but no radioactivity was observed when the corresponding fractions from the postribosomal supernatants were analyzed (Fig. 2D). 35S incorporation into MetaT1, MetβT1(R), αT5, and βT5(R) was measured, and the ratios of incorporation, MetaT1*/αT5* and MetβT1(R)*/βT5(R)*, were calculated.

The same experiments were done with sickle-cell anemia reticulocytes. Tryptic digests of ribosomal fractions labeled with [35S]methionine were prepared and analyzed on Aminex A5 and AG50W-X2 columns (Fig. 3). Strong radioactivity was observed in fractions that contained peptides βT5(S), αT5, and MetaT1(S) from the Aminex A5 column (Fig. 3A). However, no radioactivity was present in the fractions that would have contained MetaβT1(S) when tryptic digests of postribosomal fractions were analyzed (Fig. 3B). The fractions at the positions of MetaT1 and αT5 (fractions 30–40 of Fig. 3A and B) were collected from both digests and analyzed on AG50W-X2. Radioactivity was observed in these fractions at MetaT1 and αT5 of tryptic digests of nascent chains, but only at αT5 in digests of globin from postribosomal supernatant hemoglobin. The radioactive fraction assumed to be MetaβT1(S) from the Aminex A5 column was collected and chromatographed on the AG50W-X2 column. The radioactivity of this fraction eluted together with chemically synthesized MetaβT1(S). This result showed that radioactivity present in fractions with the same chromatographic properties as chemically synthesized MetaT1 and MetaT1 are present in tryptic digests of nascent globin chains from sickle-cell anemia reticulocytes and absent in digests of globin prepared from postribosomal hemoglobin. Ratios of incorporation of [35S]methionine, MetaT1*/αT5* and MetβT1*/βT5*, in tryptic digests from nascent chains of rabbit and sickle-cell anemia reticulocytes are shown in Table 1.

Incorporation of [3H]lysine into MetaβT1(R) and βT1(R) of Nascent Rabbit β-Chains. A tryptic digest of 30 mg of globin carrier added to [3H]lysine-labeled nascent chains was chromatographed on Aminex A5 as shown in Fig. 4. No ninhydrin absorbance accompanied the radioactivity at the elution position of MetaβT1(R) (fraction 50, Fig. 4) after successive analyses on Aminex A5, AG50W-X2, and Aminex A5. Thus, peptides derived from the carrier globin are absent in this zone of the chromatogram, and the radioactivity of this zone must be derived from nascent chains. The amino acid composition of fraction 50 in Fig. 4 was that of βT1(R), and the relative position of fraction 50 on the chromatogram is the same as that of chemically synthesized MetaβT1(R). The fractional incorporation of [3H]lysine, MetaβT1(R)*/[MetaβT1(R)**βT1(R)*], is given in Table 2.

Incorporation of [3H]Valine and [3H]lysine into MetaβT1(S) and βT1(S) of Nascent β-Chains of Hemoglobin S. Nascent chains labeled with [3H]valine and [3H]lysine were digested with trypsin, and the digest was chromatographed on Aminex A5. The fractions with MetaβT1(S) and βT1(S) were collected and further purified by successive analyses on AG50W-X2 and Aminex A5 columns. Fig. 5 shows the analysis of Aminex A5 at the last stage of purification. The amino acid composition of fraction 50 was that of βT1(S), and the relative position of elution of fraction 45 is the same as that of MetaβT1(S). The fractional incorporation of [3H]amino acids, MetaβT1(S)*/[MetaβT1(S)* + βT1(S)*], is given in Table 2.

**DISCUSSION**

Our results show that methionine-containing peptides not present in tryptic digests of hemoglobin appear in tryptic digests of nascent chains from both rabbit and human reticulocytes, and that these peptides are chromatographically identical to chemically synthesized peptides with methionyl residues attached to the N-termini of αT1 and βT1 sequences.

**TABLE 1. Incorporation of [35S]methionine into nascent chains**

<table>
<thead>
<tr>
<th>Reticulocytes</th>
<th>MetaT1*/αT5*</th>
<th>MetaβT1*/βT5*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>0.15</td>
<td>0.19</td>
</tr>
<tr>
<td>Sickle cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R.W.</td>
<td>0.49</td>
<td>0.95</td>
</tr>
<tr>
<td>R.W.</td>
<td>0.36</td>
<td>0.83</td>
</tr>
<tr>
<td>R.C.J.</td>
<td>0.52</td>
<td>0.81</td>
</tr>
<tr>
<td>Average</td>
<td>0.46</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* The peptides, MetaT1 and βT5, are eluted close together; therefore the contribution of MetaT1 to βT5 was corrected by use of the results of Fig. 2C.
Since $\alpha T1$ and $\beta T1$ are the N-terminal tryptic peptides of postribosomal $\alpha$- and $\beta$-chains, respectively, the methionyl sequences must come from the initiating end of nascent chains. These results confirm the conclusions of other workers (1-7) that globin-chain synthesis is initiated by methionine, and show in addition that methionine is the only residue incorporated and removed ahead of the sequences found in hemoglobin.

Rabbit and human nascent chains differ in that a higher fraction of the latter has N-terminal methionine. Because methionine is present on the shorter nascent chains, this result means either that nascent chains in human reticulocytes attain a greater length before losing methionine or that a higher fraction of human nascent chain is short. According to Hunt et al. (8) and to Luppis et al. (9), elongation of rabbit globin chains proceeds at a uniform rate; therefore, in steadystate synthesis, nascent chains of each length are present in equal numbers in a population of rabbit polysomes, and the size of the longest nascent chain with N-terminal methionine can be calculated from the data of Table 1. There are 141 residues in the $\alpha$-chain of hemoglobin from both rabbits and humans, and there are 141 possible kinds of nascent $\alpha$-chains from the dipeptide Met-Val to the completed but unreleased $\alpha$-chain. If $x$ is the length of the longest nascent $\alpha$-chain with N-terminal methionine, there are $x - 1$ kinds of N-terminal methionyl nascent chains. Of these, 6 are shorter than MetaT1; therefore, there are $x - 7$ different nascent chains that contain MetaT1. In both rabbits and humans, the C-terminal residue of $\alpha T5$ is residue 40; consequently there are 102 kinds of nascent $\alpha$-chains, including unreleased $\alpha$-chain, that contain $\alpha T5$. Hence if MetaT1* and $\alpha T5*$ are the $^{35}S$ radioactivities in these peptides from tryptic digests of nascent chains uniformly labeled with $^{35}S$-methionine,

\[
(x - 7)/102 = \text{MetaT1}*/\alpha T5*;
\]

\[
x = 7 + 102 (\text{MetaT1}*/\alpha T5*). \quad [1]
\]

In both rabbit and human hemoglobin, the $\beta$-chain has 146 residues, and the C-terminal residue of $\beta T5$ is residue 59; accordingly, the length $y$ of the longest nascent chain with N-terminal methionine is

\[
y = 8 + 88 (\text{MetaT1}*/\beta T5*). \quad [2]
\]

Equivalent but independent calculations can be made with the data of Table 2, again with the assumption of constant rate of chain elongation. MetaT1* and $\beta T1$ from nascent $\beta$-chains of rabbit hemoglobin and human hemoglobin S were analyzed in the present study. Wilson and Dintzis (1) analyzed MetaT1,2 and $\alpha T1,2$ of nascent rabbit $\alpha$-chain. $\beta T1$ and $\alpha T1,2$ contain 8 and 11 residues, respectively. It can be shown that

\[
x = 11 + 131 [\text{MetaT1,2}*/(\text{MetaT1,2}*/\alpha T1,2*)]; \quad [3]
\]

\[
y = 8 + 139 [\text{MetaT1}*/(\text{MetaT1}*/\beta T1*)]. \quad [4]
\]

$x$ and $y$ were calculated with Eqs. [3] and [4], respectively, from the data of Wilson and Dintzis (1) for $\alpha$-chain and the data of Table 2 for $\beta$-chain. It was assumed that the presence or absence of N-terminal methionine did not affect tryptic hydrolysis at the bond between Lys-8 and Ser-9 to yield MetaT1 or $\beta T1$. The chromatographic yield of the two peptides from a mixture of chemically synthesized MetaT1 and $\beta T1$ was the same. The reproducibility of results in Table 2 was superior to that of Table 1; nevertheless the results from the two independent sets of experiments are in general agreement, that if rate of elongation of nascent chains is uniform, cleavage of N-terminal methionine occurs later in elongation in sickle-cell anemia reticulocytes than in rabbit reticulocytes.

A supernatant enzyme apparently catalyzes the removal of the initiating methionine from nascent globin chains in reticulocytes (15). During the initial stages of folding, short nascent chains might assume a conformation that favors ribosomal shielding (16) of its N-terminus from supernatant enzyme or a conformation that increases resistance of its Met-Val bond to enzyme-catalyzed hydrolysis. Differences early in the folding process are possible because MetaT1 from rabbit hemoglobin and MetaT1 from human hemoglobin S differ in three of nine aminoacid positions. Although rabbit and human $\alpha T1$ are identical, the $\alpha$-chains differ from each other in five of the first 20 aminoacid positions from the N-terminus.

### Table 2. Fractional incorporation of $[^{3}H]$lysine and $[^{3}H]$valine into MetaT1

<table>
<thead>
<tr>
<th>Exp.</th>
<th>MetaT1*/(MetaT1* + $\beta T1*$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>1a 0.18 1b 0.19 2 0.20</td>
</tr>
<tr>
<td>Sickle cell</td>
<td>1 (R.C.J.) 0.32 2 (R.W.) 0.28 3 (R.C.J.) 0.33</td>
</tr>
</tbody>
</table>

### Table 3. Maximum number of aminoacid residues in N-terminal methionyl nascent chain

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ Chain ($x$)</td>
<td>(From Eq. 1)</td>
<td>(From Eq. 3)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>Sickle cell</td>
<td>49</td>
<td>45</td>
</tr>
<tr>
<td>$\beta$ Chain ($y$)</td>
<td>(From Eq. 2)</td>
<td>(From Eq. 4)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>27</td>
<td>17</td>
</tr>
<tr>
<td>Sickle cell</td>
<td>84</td>
<td>48</td>
</tr>
</tbody>
</table>

* Columns A and B: Data of Table 1 were used for these calculations. Yields of peptides were taken to be equal in column A; yields of peptides MetaT1, $\alpha T5$, MetaT1*, and $\beta T5$ were taken to be 63, 50, 75, and 40%, respectively (14), in column B. Column C: Data of Table 2 were used for these calculations; yields of MetaT1 and $\beta T1$ were taken to be equal.

$^b$ The value for rabbit $\alpha$-chain was calculated from the result of Wilson and Dintzis (1).
An alternative mechanism that would account for the findings is that whereas the rate of elongation of rabbit nascent chains is constant, elongation of human chains might be slower near the beginning than later in synthesis (17), so that the number of short nascent chains is disproportionately large. Another alternative is that there might be less enzymatic activity toward the removal of N-terminal methionine in human than in rabbit reticulocytes. Each of these alternative possibilities can be tested experimentally.

We thank Dr. A. B. Robinson for the use of his apparatus for peptide synthesis, Dr. J. Scotchler for his help and for a gift of chloromethylated resin, Mr. P. Mitsch for his help in peptide synthesis, and Mrs. G. S. Sandeen for her help in peptide and aminoacid analyses. We thank Dr. H. Koenig and Mr. B. Ashcraft for gifts of samples of blood. This investigation was supported by Grants GM 17702 and AM 14982 from the National Institutes of Health.