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Protein Chain Initiation in Rabbit Reticulocytes*

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Abstract. About 15% of the nascent α-chains isolated from *in vivo* labeled rabbit reticulocytes have methionine as their amino terminal amino acid. The methionine is predominantly on the shortest nascent chains, suggesting that methionine initiates the synthesis of rabbit hemoglobin and is rapidly removed during further synthesis.

In *Escherichia coli* *N*-formylmethionyl-tRNA[1] initiates the synthesis of each protein by binding to a 30S ribosomal subunit in response to an initiating codon in the messenger RNA.[2] The *N*-formylmethionine (fMet) reacts with the next amino acid encoded in the message to start the synthesis of the polypeptide chain.[3,4] The fMet attached to the growing polypeptide chain is cleaved during synthesis to uncover the amino-terminal amino acid of the finished protein. In this cleavage, either the formyl group alone or the formyl group and the methionine are removed, depending on which protein is being synthesized.[5]

In higher organisms, fMet-tRNA has never been found except in mitochondria[6,7] and chloroplasts,[8] where it seems to function as the initiator of protein synthesis within these organelles. One cytoplasmic system in which initiation has been studied is the biosynthesis of the α- and β-chains of hemoglobin in rabbit reticulocytes. In these cells, about 95% of the protein synthesized consists of the α- and β-chains of hemoglobin. Thus, it is possible to examine the incomplete chains bound to the ribosomes and try to discover if they are modified at their amino terminus.

Rahaminoff and Arnstein[9] have determined the percentage of [^{14}C]valine, the amino-terminal amino acid of the α- and β-chains, incorporated into reticulocyte ribosomes which could be deaminated by nitrous acid. By making an assumption as to the average length of the incomplete chains, they concluded that all of the incomplete chains have free valine at their amino terminus.

Mosteller, Culp, and Hardesty[10] determined the extent and kinetics of the incorporation of [^{14}C]acetate and [^{14}C]formate into reticulocyte ribosomes. They found that both the rate and extent were very low and not compatible with a role for either in initiation. They also measured the amount of [^{14}C]-valine incorporated into reticulocyte ribosomes which could react with dinitrofluorobenzene. They concluded that most or all of the incomplete chains have free valine at their amino-terminal end. Finally, they found that the poly U directed synthesis of polyphenylalanine on reticulocyte ribosomes was greatly stimulated by deacylated tRNA at 4 mM $MgCl_2$. From all their data, they be-

lieve that the mechanism of initiation in hemoglobin biosynthesis may involve the binding of deacylated tRNA to phase the message, with direct incorporation of valine at the amino terminus of the growing polypeptide chain.

Gonano and Baglioni[11] labeled reticulocyte ribosomes with a mixture of [^{14}C]-amino acids and looked for the presence of amino acids with a blocked amino group by digesting the nascent chains with pronase or with trypsin and carboxypeptidase B, running the digests through a Dowex 50 column and counting the effluent. In neither case did they find a significant amount of ^{14}C in the effluent. Thus, they concluded that the initiation of hemoglobin biosynthesis does not require a blocked amino acid. They also showed that both reticulocyte and *E. coli* tRNA charged with [^{14}C]valine could transfer the label into the amino terminal position of hemoglobin in a reticulocyte cell-free system. From their experiments, they concluded that cytoplasmic ribosomes do not require a blocked amino group to initiate the synthesis of proteins, and can function with different amino acids as the initiator.

Since previous attempts to find a natural initiator for hemoglobin synthesis had failed, we attempted to design a highly specific and sensitive assay for nascent polypeptide chains transiently modified at their amino end.[12] Such an assay is possible because of the low frequency and fortunate placement of isoleucine residues in rabbit hemoglobin. We have observed that in some rabbits there is no isoleucine in the β-chain. The α-chain contains three isoleucine residues: at positions 10 (in tryptic peptide 2, i.e. αT2), 17 (in tryptic peptide 4, i.e. αT4), and 55 (in tryptic peptide 6, i.e. αT6).[13] From a complete tryptic digest of total rabbit hemoglobin from an animal containing no isoleucine in the β-chain, one would therefore expect to find only three peptides containing isoleucine–αT2, αT4, and αT6. Actually, since the tryptic split at the lysine in position 7 is incomplete, one additional isoleucine-containing peptide is found in high yield, αT 1 + 2 (Fig. 1). This peptide contains the amino end of the

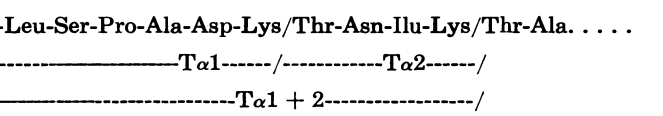

FIG. 1. Sequence of tryptic peptide αT 1 + 2 from rabbit hemoglobin.

α-chain and a low-frequency amino acid and so it can be labeled quite specifically by short incubations of living cells with [^{14}C]isoleucine. Accordingly, experiments were designed to determine whether incomplete ribosome-bound polypeptide chains of hemoglobin contained a transiently modified form of αT 1 + 2.

We have found that 15% of this amino-terminal peptide from the nascent α-chains occurs as a separable derivative with the structure MetVal..., while the remaining 85% occurs as αT 1 + 2 with its amino-terminal valine residue unmodified.

Materials. New Zealand white rabbits were screened to obtain those which lack isoleucine in their β-chain. Reticulocytes were obtained by bleeding rabbits made anemic by five 1-ml injections of 2.5% phenylhydrazine given one each day. Dowex 50 (aminex X_2) was obtained from Biorad Laboratories, while DEAE-cellulose (DE 32)

was obtained from Whatman. Trifluoroacetic acid and phenylisothiocyanate were redistilled and stored at −20°C. All other chemicals used in the Edman degradation were chromoquality reagents obtained from Matheson, Coleman and Bell. *N*-ethylmorpholine was obtained from Eastman Kodak and redistilled.

Labeled ribosomes were prepared by incubating 8 ml of packed reticulocytes, washed twice with 0.14 M NaCl, 1 ml of 2% glucose, 1 ml of 1.5 mM $FeNH_4SO_4$, 8 ml of saline (0.14 M NaCl, 2 mM $MgCl_2$, 10 mM Tris, pH 7.5), and 1 ml of an amino acid mixture containing (per milliliter) 1.9 mg His, 1.3 mg Phe, 0.9 mg Ser, 1.3 mg Lys, 1.4 mg Trp, 0.7 mg Tyr, 1.4 mg Gln, 1.4 mg Asn, 2.6 mg Leu, and, when needed, 1.9 mg Val for 10 min at 35°C; then the labeled amino acids were added and the incubation was continued for 5 min. The incubation mixture was chilled, 15 ml of saline at 4°C were added, and the mixture was centrifuged at 10,000 × *g* for 10 min at 4°C. The cells were lysed in 10 ml of 2 mM $MgCl_2$ and centrifuged at 15,000 × *g* for 15 min, the supernatant was carefully removed, the pellet was washed with 6 ml of 2 mM $MgCl_2$ and centrifuged, and the supernatants were combined. Three-ml aliquots of the lysate were layered over 6 ml of 30% sucrose containing 2 mM $MgCl_2$, 10 mM KCl, 10 mM Tris (pH 7.5) in a 40 rotor polycarbonate tube and centrifuged at 39,000 rpm for 3 hr in the 40 rotor of the Spinco ultracentrifuge. The supernatant was removed and the pellets were rinsed with H_2O, resuspended in 3 ml of H_2O, and frozen. Globin was prepared by adding 10 vol of −20°C acetone containing 0.1% concentrated HCl and 0.1% mercaptoethanol to 1 vol of lysate.

Tryptic digestions were performed on about 10 mg of ribosomes mixed with 10 mg of globin obtained from the same rabbit as the ribosomes, using 0.2 mg of trypsin. This mixture was incubated at 37°C for 3 hr at pH 9.0 in a pH stat. Then the pH was adjusted to 6 with 1 M acetic acid and the precipitate removed by centrifugation. Sephadex G25 chromatography of the tryptic peptides was conducted on a 1.5 × 81 cm column equilibrated with 20 mM NH_4OAc, 1 mM NaN_3. The column was run at room temperature and 2.5-ml fractions were collected. Fractions 24–29, which constituted the major ^{14}C peak from the column and contained peptides αT 1 + 2 and αT4, were combined and lyophilized.

DEAE-cellulose chromatography was performed on a 2 × 15 cm column using a three-chamber gradient with 60 ml of buffer B (7.5 ml pyridine, 3.75 ml *N*-ethylmorpholine, 0.5 ml of 1 M acetic acid) in the first chamber, 60 ml of buffer B adjusted to pH 8.5 with acetic acid in the second chamber, and 60 ml of buffer B adjusted to pH 6.5 in the third chamber. Dowex 50 chromatography was conducted on a 1 × 60 cm column equilibrated with buffer L (8 ml pyridine plus 15 ml glacial acetic acid to 500 ml) using a three-chamber gradient containing 100 ml buffer L in the first chamber, 90 ml buffer L, 10 ml buffer H (25 ml glacial acetic acid plus 40 ml pyridine to 250 ml) in the second chamber, and 30 ml buffer L, 70 ml buffer H in the third. Fractions of 2.6 ml were collected and 0.2-ml aliquots were counted.

The Edman degradation was performed by the procedure of Doolittle[14] except that all quantities were reduced by a factor of 10. All radioactivity measurements were made in a Packard tricarb scintillation counter using 10 ml of triton toluene counting solution (1 liter toluene, 500 ml triton, 7.5 g PPO, and 9.4 mg POPOP).

Results. Identification of a modified amino-terminal peptide: Ribosomes labeled with [^{14}C]isoleucine were mixed with unlabeled globin and digested with trypsin, the digest was chromatographed on G25 Sephadex, and the peak which contained αT 1 + 2 was chromatographed on Dowex 50. Figure 2*A* shows the results of the Dowex 50 column, while Figure 2*B* shows the results of a second experiment in which [^{14}C]globin and unlabeled ribosomes obtained from the same rabbit as before were used. Aliquots of all peaks were hydrolyzed and run on a Beckman amino acid analyzer. The bulk of the peptide material analyzed in this way is derived from the added globin. Peak I had the amino acid composition of αT 1 + 2 and peak III of αT4. Looking at the radioactivity in these

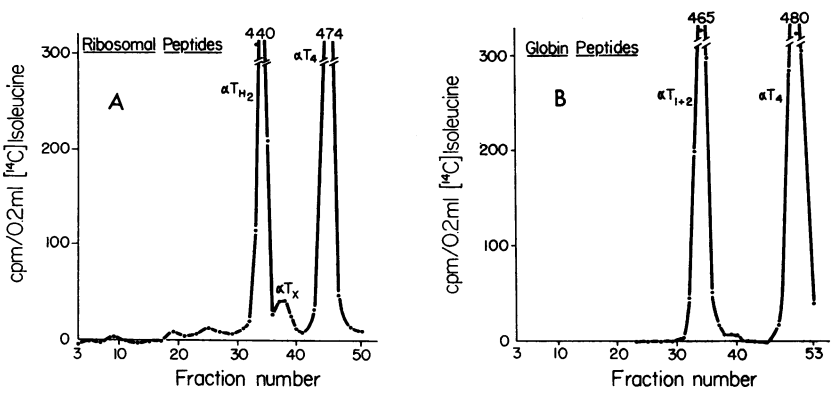

FIG. 2. Column chromatography on Dowex 50 of globin tryptic peptides. (*A*) Tryptic peptides from [^{14}C]-isoleucine-labeled ribosomes plus unlabeled globin. (*B*) Tryptic peptides from [^{14}C]-isoleucine-labeled globin and unlabeled ribosomes obtained from the same rabbit as (*A*).

peaks, we see that labeled ribosomes (Fig. 2*A*) yield αT 1 + 2 and αT4, showing that the label is indeed in globin intermediates, and that since αT 1 + 2 is the amino-terminal peptide of globin, many of the growing chains have the same amino-terminal sequence as the completed globin molecules. That the ^{14}C αT 1 + 2 does have *N*-terminal valine is shown in Table 3. Thus, we find that in agreement with other workers, most of the growing chains lack any special blocking group.

There is, however, a small peak of isoleucine-containing peptide from the labeled ribosomes (peak II, Fig. 2*A*, designated αTx) which is not present in significant amount in the completed labeled globin. This peak has been found in every ribosomal digest (25 in all) which has been examined. It does not correspond to any globin peptide, so analysis of peak II in the Beckman amino acid analyzer shows only trace amounts of amino acids. αTx is closely related to αT 1 + 2 in chromatographic behavior and thus seems to be the modified amino terminal peptide for which we are looking.

Since αTx is only present in incomplete ribosome-bound chains and is present on only a fraction of them, we obtain at best 10^{-10} moles of αTx per rabbit. This means that we cannot determine its structure by normal chemical and physical procedures. Instead, we have used an isotopic method to determine the amino acid composition of αTx. Ribosomes labeled with a mixture containing all 20 [^{14}C]amino acids except cysteine were digested and αT 1 + 2 and αTx were isolated as before except that the peak from the G25 Sephadex column was first chromatographed on the DEAE column. The first peak from this column was lyophilized and chromatographed on the Dowex 50 column. The purified αT 1 + 2 and αTx were hydrolyzed, after carrier amino acids were added to αTx, for 24 hr at 110°C in 6 N HCl, dried in a desiccator, and separated by electrophoresis on 3 MM paper at pH 1.9 for 3 hr at 4,500 V. The region containing the neutral and acidic amino acids was cut out, sewed to Whatman no. 1 paper, and chromatographed at right angles to the direction of electrophoresis using

the solvent system methyl ethyl ketone, propionic acid, water, 15:5:6. The rest of the electrophoresis and the chromatogram were dried and stained with 0.02% ninhydrin, and the amino spots were cut out and counted in a scintillation counter. All amino acids were separated by this procedure except leucine and isoleucine, which were counted together.

This same procedure was also applied to a sample of globin prepared from the same incubation. The results of several such experiments are presented in Table 1. This method gives the correct composition for αT 1 + 2 and shows that αTx is almost identical in amino acid composition to αT 1 + 2. However, in this experiment, the specific activity of methionine was too low to be certain that it is missing from αTx. Therefore, we ran an incubation using [^{14}C]-isoleucine and high specific activity [^{3}H]methionine. Table 2 gives the results of two such incubations, which indicate that αTx contains one residue of methionine. The ^{3}H and ^{14}C counts in αTx coelectrophoresce at pH 6.5, providing further evidence that they are in the same peptide.

If methionine is the initiator, then it should be the amino-terminal amino acid in αTx. To check this, [^{3}H]methionine-labeled αTx was subjected to the Edman degradation with the results given in Table 3. These show that methionine is the amino-terminal amino acid of αTx and that its amino group is not modified in any way. The ^{3}H-labeled PTH derivative from methionine-labeled αTx cochromatographed with PTH methionine in xylene and in a butyl acetate, propionic acid solvent. αTx labeled with [^{14}C]valine was subjected to two cycles of the Edman degradation (Table 3). This last experiment indicates that valine is the penultimate amino acid in αTx and that αTx thus is identical to αT 1 + 2 except for an additional methionine residue at its amino-terminal end.

TABLE 1. *Amino acid composition of αTx and αT 1 + 2 from nascent chains determined isotopically.*

Amino acid	^{14}C in αTx (cpm)	^{14}C in αT 1 + 2 (cpm)	Residues in αTx	Residues in αT 1 + 2
Lysine	163	428	1.9	1.8
Histidine	0	0	0	0
Arginine	4	12	0.01	0.02
Aspartic acid	187	428	1.9	1.6
Threonine	83	203	0.9	0.9
Serine	103	294	1.0	1.2
Glutamic acid	14	5	0.03	0.03
Proline	18	37	1.2	0.9
Glycine	4	0	0.2	0
Alanine	39	70	1.0	1.1
Valine	121	253	1.0	1.0
Methionine	5	0	0.3	0
Isoleucine plus leucine	176	865	1.8	3.3
Phenylalanine	0	2	0	0.01
Tyrosine	0	4	0	0.02
Tryptophan	0	0	0	0

Values of cpm are from a typical run while the values of residues are the averages of three runs. The values for residues were calculated using the relative specific activity of each amino acid measured in globin isolated from the same incubation, with all values in the peptides normalized to valine as 1.0 residue.

TABLE 2. *Methionine content of αTx.*

Expt.	^{3}H-Met (cpm)	^{14}C (cpm)	Residues Met
1	400	70	0.77
2	320	113	0.78

αTx in Expt. 1 was isolated from ribosomes labeled with [^{14}C]isoleucine and [^{3}H]methionine. The residues of methionine were calculated using the relative specific activity (*SA*) of methionine to isoleucine in the globin isolated from the incubation (determined by counting it and running an amino acid analysis on it) using the formula:

$$\text{SAMet/SAIlu} = \text{cpmMet/cpmIlu} \cdot \text{residues Ilu/residues Met}$$

αTx in Expt. 2 was isolated from ribosomes labeled with [^{14}C]valine and [^{3}H]methionine. Residues methionine were calculated using the *SA* of Met to Val found by counting BT_5 isolated from the same ribosomal digest as αTx. BT_5 contains one residue of Met and one of Val.

TABLE 3. *Edman degradation of αTx.*

Expt.	Peptide	Cpm Initially ^{3}H	Cpm Initially ^{14}C	Cpm PTH Fraction ^{3}H	Cpm PTH Fraction ^{14}C	Percentage of Release Met	Percentage of Release Val
1	αTx	358	63	288	0	80	—
1	αTx	570	100	362	0	64	—
2	αTx	960	330	640	7	67	2
2	αTx	960	330	627	20	65	6
2	αTx 2nd cycle	120	98	6	64	5	65
2	αTx 2nd cycle	180	128	53	92	29	72
2	αT 1 + 2	23	890	0	706	—	79

Expts. 1 and 2 were described in Table 2.
αTx 2nd cycle was the peptide residue from the first Edman degradation.

Table 3 also gives the results of experiments in which αT 1 + 2 labeled with [^{14}C]valine was subjected to one cycle of the Edman degradation. The release of valine confirms that the labeled ribosomal peptide in peak I (Fig. 2*A*) is indeed unmodified αT 1 + 2.

If methionine is initiating the synthesis of the α-chains and is then being cleaved during synthesis, one should find more αTx in digests of short incomplete chains than in digests of long incomplete chains. This was tested in the following experiment. Labeled ribosomes were incubated at pH 11.0 for 3 hr at 37°C to release the incomplete chains from tRNA. The mixture was then run over a G-75 column in urea at pH 9. The material in the first two thirds of the polypeptide fractions was concentrated by ultrafiltration to give the large incomplete chains. The material in the last third was concentrated to give the short incomplete chains. Carrier globin was added to each sample which was digested and the tryptic peptides were fractionated as before. The results of two such experiments are given in Table 4 and show that, as was predicted, αTx is almost completely derived from the short incomplete chains.

Discussion. All these experiments provide strong evidence that methionine serves to initiate the synthesis of the α-chain of hemoglobin and is rapidly removed during further synthesis of the α-chain. These results do not apply directly to other systems; however, they provide strong support for other experiments which suggest that methionine can serve as the initiator of cytoplasmic protein synthesis in higher organisms. Yeast has been shown to contain two species of Met tRNA.[15] One of these, called tRNAf, resembles the initiating

TABLE 4. *αTx content of tryptic digests of long and short nascent chains.*

Expt.	Sample	αTx ³H (cpm)	αTx ¹⁴C (cpm)	αT 1 + 2 ³H (cpm)	αT 1 + 2 ¹⁴C (cpm)	Total cpm ¹⁴C in αTx (%)
1	Short chains		4,500		12,000	27
	Long chains		0		7,000	0
2	Total nascent chains	3,300	650	480*	4,000	14
	Short chains	4,160	800	900*	900	47
	Long chains	484	160	0	1,800	8

In expt. 1 the nascent chains were obtained from [¹⁴C]isoleucine-labeled ribosomes, while in expt. 2 they came from [³H]methionine-, [¹⁴C]isoleucine-labeled ribosomes.

* These counts represent about 0.02 and 0.2 residues methionine in the αT 1 + 2 peaks from total and short chains, respectively.

Met tRNA of *E. coli* in its chromatographic properties and in being a substrate for the *E. coli* transformylase.[16] Wheat embryos, beef liver,[17] and guinea pig liver[18] also seem to contain a similar pair of methionine tRNA's. It has been shown that yeast Met tRNAf cannot incorporate methionine into internal positions either in an *E. coli* or a reticulocyte cell-free system, while the other species of yeast Met tRNA can incorporate methionine internally.[19,20] This suggests that the yeast Met tRNAf species functions in chain initiation and the presence of similar Met tRNA's in several different organisms indicates that this mechanism may be widely used. Such a tRNA may be responsible for the initiation of the α-chain of hemoglobin reported here. That methionine, not *N*-formyl or some other acylated derivative of methionine, is the initiator is suggested by the fact that the initiator peptide, αTx, has a free amino group and that Marcker and Sanger[1] found no sign of any acylated methionine on *in vivo* charged cytoplasmic yeast tRNA.

Experiments are in progress to see whether methionine also initiates the synthesis of the β-chain of hemoglobin and to look for the enzyme which removes methionine from the nascent chains.

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