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Cotranslational heme binding to nascent globin chains

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Globin synthesis in cell-free extracts of rabbit reticulocytes was carried out in the presence of ^{3}H-labeled hemin. Sucrose gradient centrifugation analysis revealed [^{3}H]hemin in the polyribosome fraction. The addition of puromycin resulted in the release of both [^{3}H]hemin- and [^{14}C]leucine-labeled polypeptide from the polyribosomes. The data suggest cotranslational folding of the globin molecule on the ribosome and cotranslational heme binding to the nascent globin chain.

Hemoglobin; Heme; Attachment; Nascent peptide; Cotranslational folding

1. INTRODUCTION

Various proteins have been shown to bind ligands. Various derivatives of adenosine, glucose, porphyrin and α-amino acids are typical examples of such cofactors [1]. Ligand binding was shown to proceed posttranslationally for several proteins, e.g. cytochromes *c* [2]. On the other hand, studies of biosynthesis of protein D1 of the membrane-bound chloroplast reaction center suggested cotranslational binding of chlorophyll to D1 [3,4]. Cotranslational binding had also been assumed to take place for the heme group in the case of globins [5]. In this communication an attempt is made to determine directly, by the use of [^{3}H]labeled hemin, whether heme attachment proceeds co- or post-translationally. The results obtained indicate that heme attachment proceeds cotranslationally to a nascent globin chain still attached to the ribosome. This also suggested cotranslational folding of the globin molecule.

2. MATERIALS AND METHODS

2.1. *Hemin labeling*

^{3}H-labeled hemin was prepared by the hot tritium bombardment technique [6,7]. Hemin (Koch Light) (8 mg) was diluted in 1.5 ml of 0.01% NH_4OH. The solution was layered on the inner wall of the glass reactor and lyophilized overnight. Bombardment was done for 10 min at a tritium gas ([$^3H_2$]) pressure of 10^{-3} torr. Tritium was injected in doses using an ampoule with a total radioactivity of 0.1 Ci. Tritium atoms were generated in the reactor by heating the tungsten wire to 2,000K with an alternating current. After bombardment, hemin was dissolved in 15 ml of 0.01% NH_4OH and then precipitated by the addition of 400 μl CH_3COOH. To remove labile bound tritium the procedure of solution and precipitation was repeated 17 times. After that the hemin solution was lyophilized. 1 mM hemin solution in 90% ethyleneglycol (specific radioactivity 1.2 mCi/ml) was prepared as described in [8] and used in the translation experiments.

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2.2. *Cell-free protein synthesis*

Rabbit reticulocyte lysate (untreated) was a gift of P. Simonenko (Institute of Protein Research, Pushchino, Russia). In vitro translation was performed under standard conditions at 34°C as described by Pelham and Jackson [9] in the presence of [^{14}C]leucine (1 μCi/ml, Amersham) and [^{3}H]hemin (if necessary). Creatine phosphokinase and nonlabeled hemin were added during preparation of the lysates at final concentrations of 0.1 mg/ml and 30 μM, respectively. After 3 or 5 min of incubation, the reaction mixtures were subdivided into two equal portions. An equal volume of cold (in ice) buffer A (10 mM Tris-HCl, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA) was added to one of the portions to stop the translation. Puromycin was added to a final concentration of 1 or 1.5 mM to the second portion; in this case the incubation was continued for an additional 5 or 7 min followed also by addition of an equal volume of buffer A.

2.3. *Sucrose gradient centrifugation*

After incubation, the extracts were layered on the top of linear 0.5 to 1.5 M sucrose gradients in buffer A. Centrifugation was done for 2 h at 35,000 rpm in an SW41 rotor at 4°C. The gradients were pumped from the bottom and the absorbance at 279 nm were continuously recorded. Approximately 400 μl fractions were collected and the radioactivity was counted.

3. RESULTS AND DISCUSSION

We examined rabbit reticulocyte polyribosomes obtained during globin synthesis (in the presence of labeled hemin) in the cell-free system in order to determine whether heme attachment proceeds cotranslationally. The hot tritium bombardment technique [6,7] was applied to label the hemin. The spectral properties of the hemin obtained after tritium bombardment and purification were closely similar to those of unlabeled hemin. A twenty-three percent excess of the ^{3}H-labeled hemin added to the translation mixture containing an optimal concentration (30 μM) of unlabeled hemin did not sig-

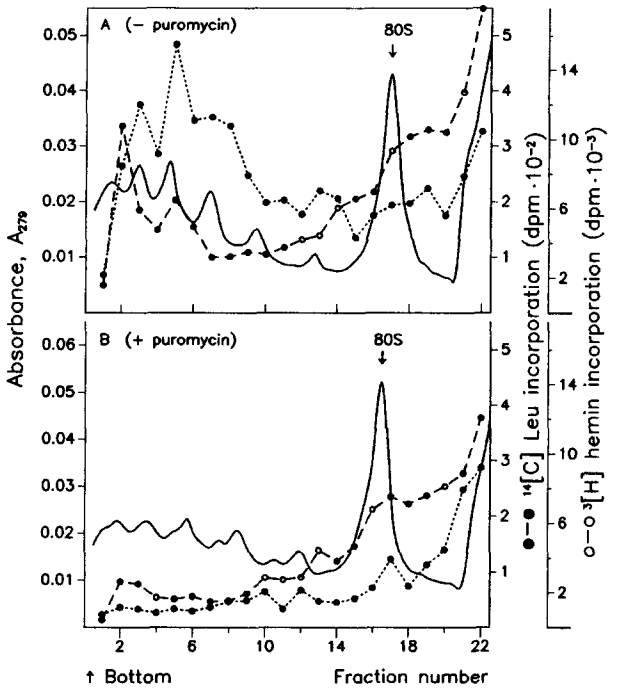

Fig. 1. Incorporation of [^{14}C]leucine and [^{3}H]hemin into the polyribosome fraction of rabbit reticulocyte lysates. (A) No antibiotic was added. (B) After the addition of puromycin. The last seven fractions from the top of the gradients are excluded from the consideration here because of the high values of radioactivity measured and for a better visualization of the data obtained.

nificantly alter protein synthesis. The incorporation of the radioactive amino acids in this case was linear for at least 20 min (at 34°C) (data not shown). The incorporation of [^{3}H]hemin into the de novo synthesized globin chains was also shown by gel filtration and by electrophoretic analysis of the cell-free translation products (data not shown). Sucrose gradient centrifugation analysis of rabbit reticulocyte lysates revealed detectable amounts of ^{3}H-labeled hemin associated with the polyribosome fraction. To determine whether heme is attached to peptidyl-tRNA, 1 or 1.5 mM of puromycin was added to the incubation mixture after 3 or 5 min from the beginning of globin synthesis, and incubation was continued for additional 5 or 7 min. No detectable amounts of either [^{14}C]leucine or [^{3}H]hemin were found in the polyribosome fraction in contrast to the experiment without puromycin (Fig. 1a,b). This indicates that heme is attached to the nascent globin chains on the ribosome. The slight difference in polyribosome profiles between the experiments with and without puromycin can apparently be ascribed to a partial dissociation of the ribosomal subunits from mRNA under conditions of puromycin treatment leading to the release of peptidyl-tRNA.

We have found [^{3}H]hemin to be predominantly associated with polyribosomes containing five to six ribosomes. This suggests that the heme incorporation occurred at the final stages of globin synthesis, apparently after the moment of appearance of both histidine imidazole groups of the F and E helixes necessary for heme attachment [10]. In accordance with this assumption it has been found that the large proteolytic fragment of the β-globin molecule comprising residues 31 to 104 is capable of binding the heme group [11]. Taking into account that the ribosome protects about 30 amino acid residues of the nascent peptide [12–15], it can be assumed that the nascent peptides of 100 amino acid residues and longer (i.e. when at least 70 amino acid residues are extending from the ribosome) are capable of binding the heme groups.

It is widely believed that formation of the hemoglobin tetramer takes place posttranslationally. Nevertheless, the possibility of tetramer assembly on the ribosome cannot be completely excluded. If this is the case, the data obtained in this study could also be explained by the presence of the three complete globin chains (containing labeled hemin) associated with a nascent chain. This could mean that the nascent chain possesses a spatial structure allowing its interaction with the globin trimer and thus does not exclude the possibility of hemin incorporation into the incompleted subunit.

The experiments reported here suggest cotranslational folding of the globin molecules. It is known that denatured globin or myoglobin molecules are unable to bind the heme group. It is also known that the detachment or displacement of heme groups may be accompanied by denaturation of the hemoglobin, while the addition of the heme group to apohemoglobin or apomyoglobin promotes the formation of the native structure of molecules [16]. Hence, it can be assumed that the formation of the globin spatial structure can proceed on the ribosome and that heme attachment stimulates the cotranslational formation of the protein tertiary structure.

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