Proc. Nat. Acad. Sci. USA
Vol. 71, No. 8, pp. 2988–2991, August 1974

Acetylation of Reticulocyte Ribosomal Proteins at Time of Protein Biosynthesis

(sodium fluoride/cyclohexamide)

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Communicated by Charles H. Best, May 6, 1974

ABSTRACT **When rabbit reticulocytes were incubated *in vitro* with [³H]acetate, their ribosomal proteins were rapidly acetylated within 10 min. Polyacrylamide–urea gel electrophoresis showed that several major ribosomal protein fractions were highly acetylated. By the double-isotope labeling technique, the incorporation of [³H]acetate and [¹⁴C]aminoacid mixture into ribosomal proteins and nascent chains was found to be closely associated. Sodium fluoride abolished the acetylation of ribosomal proteins, whereas cycloheximide reduced the acetylation of ribosomal proteins to a much lower level. These findings suggest that acetylation of ribosomal proteins may be involved in the formation of the initiation complex during protein biosynthesis.**

The postsynthetic modification of protein structure at the transcriptional level by acetylation, phosphorylation, and methylation has been implicated in the regulation of cellular activity (1). Allfrey and his coworkers (2, 3) have provided the first evidence that there is a close temporal interrelationship between an induced RNA synthesis and covalent modification of histones. Dixon and his coworkers (4, 5) have also suggested that covalent modification of histone in trout testis is an obligatory step toward the assembly of chromatin.

Recently we have found (6) that ribosomal proteins are acetylated *in vivo* as well as *in vitro*, and we have also shown that liver ribosomal proteins are phosphorylated, an observation similar to that of Kabat (7) on reticulocyte ribosomal proteins and that of Eil and Wool (8) on rat liver ribosomes. We have suggested (9) that covalent modification of proteins at both transcriptional and translational sites may involve a common mechanism to regulate gene activation and protein biosynthesis. The experiments described here are designed to study the nature of the regulatory function of ribosomal proteins in relation to protein biosynthesis in reticulocytes.

MATERIALS AND METHODS

All chemicals and organic solvents used in these studies are of reagent grade. *N,N,N′,N′*-Tetramethylenediamine, acrylamide, and *N,N′*-methylenebisacrylamide were purchased from Eastman Organic Chemicals; sucrose and urea were from Schwarz/Mann; NCS solubilizer was from Amersham/Searle; and Liquifluor was from New England Nuclear. [³H]Acetate (specific activity 3.2 Ci/mmol) and [¹⁴C]aminoacid mixture were purchased from Amersham/Searle.

Rabbits were made anemic by injection of phenylhydrazine as described by Marbaix and Burny (10). More than 90% of the blood cells were reticulocytes, as demonstrated by staining with brilliant cresyl blue. The reticulocytes were washed three times with an ice-cold physiological salt solution containing 130 mM NaCl, 5 mM KCl, and 7.5 mM $MgCl_2$ in 10 mM Tris·HCl buffer, pH 7.5. The washed reticulocytes were then suspended in the same medium containing 5% (v/v) normal rabbit serum and 5 μg/ml of actinomycin D. The cell suspensions contained 2 to 3 × 10^9 cells per ml. All media used in these studies had been filtered through a Millipore membrane before use.

Isolation of Ribosomal Proteins. Ribosomes were prepared from the reticulocyte lysate. The intact reticulocytes were first lysed with a solution containing 10 mM Tris·HCl buffer (pH 7.4), 10 mM KCl, and 1.5 mM $MgCl_2$. The 10,000 × *g* supernatant of the lysate was then layered onto 1 M sucrose containing 0.5 M KCl, 10 mM $MgCl_2$ in 0.1 M Tris·HCl, pH 7.4. The ribosomal pellets were obtained by centrifugation at 198,000 × *g* for 90 min as described (11). The ribosomal proteins were obtained by extraction with LiCl–urea. In some experiments, the ribosomes were treated with puromycin in concentrated salt (0.5 M KCl) solution to remove nascent chains before isolation of ribosomal proteins as described (6).

Isolation of Nascent Chains. Ribosomes were first treated with puromycin *in vitro* in a high salt concentration at 37° and subsequently layered onto 1 M sucrose in 0.5 M KCl–10 mM $MgCl_2$–0.1 M Tris·HCl through centrifugation at 198,000 × *g* for 4 hr. The nascent chains were then isolated from the supernatant by precipitation with 10% trichloroacetic acid.

Sucrose Density Gradients. Five absorbance units of ribosomes at 260 nm were layered on top of 5 ml of 10–30% linear sucrose density gradient in Medium N (12). The ribosomes were centrifuged at 178,000 × *g* for 30 min in an SW 50.1 rotor (Spinco). Fractions were collected, and radioactivity was determined as described (6).

Polyacrylamide–Urea Electrophoresis. Ribosomal proteins obtained from LiCl–urea extraction after puromycin treatment to remove the nascent chains were fractionated by 10% polyacrylamide–urea gel electrophoresis (13). The gels were stained with 0.2% Amido Black and destained electrophoretically in 7% acetic acid. Gels were scanned at 570 nm with an attachment to a Unicam SP 1800 spectrophotometer (14).

Identification of Acetyl Groups in Ribosomal Proteins. Ribosomal proteins obtained from LiCl–urea extraction were precipitated with 10% trichloroacetic acid. They were then dissolved in 0.5 M NaOH and hydrolyzed at 37° for 30 min to remove any possible contamination with ribosomal RNA. The ribosomal proteins were then precipitated with 10% trichloroacetic acid and exposed to ethanol–ether (1:1) and ether to remove any phospholipid residues. These proteins

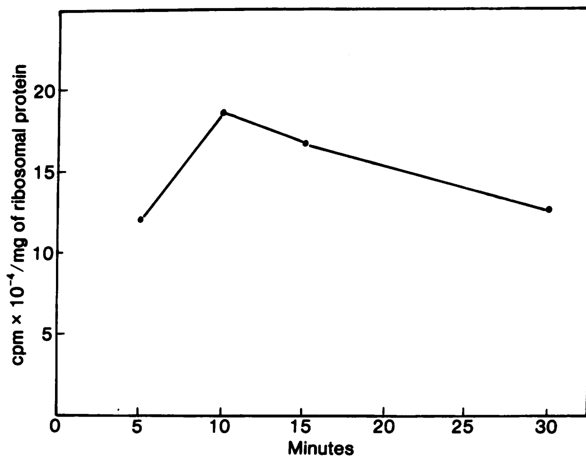

FIG. 1. Reticulocyte suspension (50 ml) containing 2 × 10^9 cells per ml was incubated at 37° for 5 min before addition of 2.5 mCi of [^{3}H]acetate (specific activity, 2.7 Ci/mmole). Aliquots of 10 ml were taken at 5, 10, 15, and 30 min after addition of [^{3}H]-acetate. The reaction was stopped by cooling in ice and addition of 20 ml of ice-cold physiological salt solution (*Materials and Methods*). The samples were immediately pelleted at 6,000 × *g* for 5 min in a Sorvall RC-2B refrigerated centrifuge. The reticulocytes were lysed in 10 mM Tris·HCl-10 mM KCl-1.5 mM $MgCl_2$. The ribosomal proteins were then isolated as described in the *text*.

were hydrolyzed in 6 M HCl at 110° under reduced pressure for 18 hr. The acetate was subsequently removed by flash evaporation as described (6).

RESULTS

Incorporation of [^{3}H]Acetate into the Ribosomal Proteins. Fig. 1 shows that the reticulocyte ribosomal proteins were rapidly labeled with [^{3}H]acetate within 10 min. Hydrolysis of the [^{3}H]acetate-labeled ribosomal proteins with 6 M HCl at 110° for 18 hr resulted in over 70% of the radioactivity being volatile. The volatility of the radioactivity was also confirmed by steam distillation in the presence of H_2PO_3. It is, therefore, evident that the [^{3}H]acetate was covalently incorporated into the ribosomal proteins as a result of acetylation.

Characterization of Acetylated Ribosomal Proteins by Polyacrylamide–Urea Gel Electrophoresis. The acetylated ribosomal proteins that were obtained from the intact reticulocytes during 10-min pulse-labeling *in vitro* were fractionated by polyacrylamide–urea gel electrophoresis. The specific activities of some major bands of the fractionated ribosomal proteins were determined by elution of the dye from the stained proteins and determination of the radioactivity in these proteins as described (15). Results are shown in Fig. 2 and Table 1. All ribosomal proteins were acetylated to different extents. However, the ribosomal proteins in band 10 plus 11 were the most highly acetylated.

Causal Interrelationship of Acetylation of Ribosomal Proteins and Protein Biosynthesis. An attempt was made to examine the causal interrelationship between acetylation of ribosomal proteins and protein biosynthesis by using NaF and cycloheximide as inhibitors of protein synthesis. A suspension of reticulocytes (2.6 × 10^9 cells per ml) was incubated with both [^{3}H]acetate and [^{14}C]aminoacid mixtures for various intervals. NaF or cycloheximide markedly inhibited the acet-

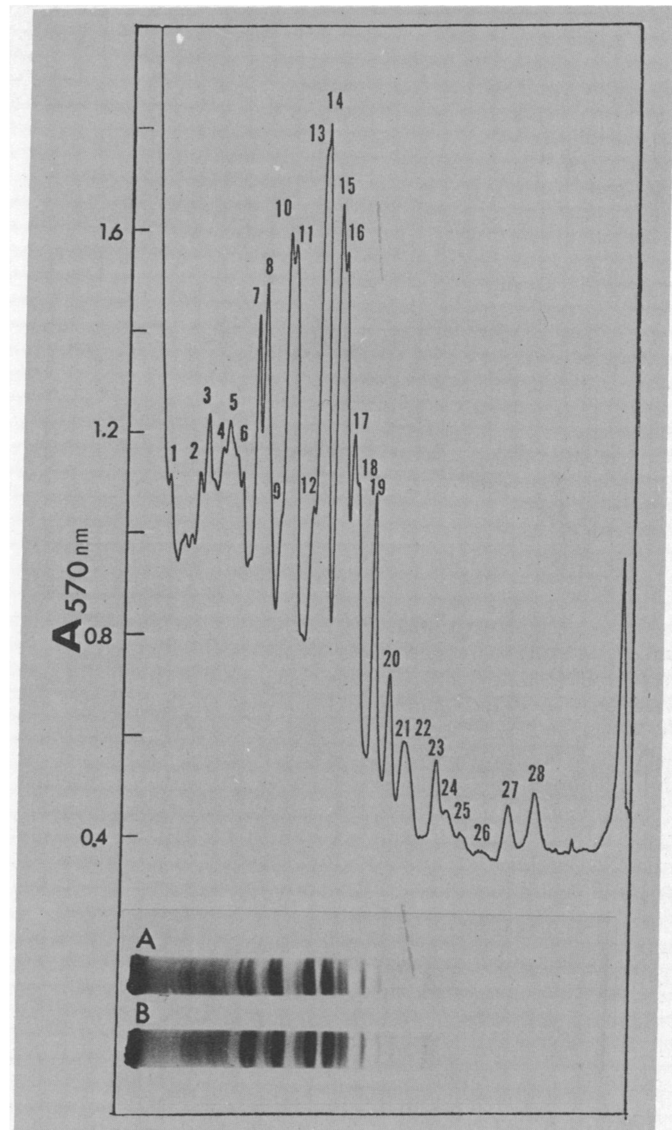

FIG. 2. Ribosomal proteins obtained from LiCl–urea extraction were fractionated by polyacrylamide–urea gel electrophoresis (13). The gels were stained with 0.2% Amido Black and destained electrophoretically. The gels were then scanned at 570 nm. (*A*) Proteins from ribosomes that had been treated with puromycin in the presence of high salt concentration; (*B*) proteins from ribosomes that were directly extracted by LiCl–urea. Both showed identical electrophoretic patterns.

ylation of ribosomal proteins and protein synthesis when the inhibitor was added to the cell suspension 5 min after the incubation had begun (Fig. 3). However, the inhibitory effect of cycloheximide on the acetylation of ribosomal proteins was relatively less than that of NaF.

When the cell suspensions were incubated with NaF or cycloheximide for 5 min at 37° before addition of [^{3}H]acetate and [^{14}C]aminoacid mixture, the incorporation of [^{3}H]acetate into ribosomal proteins was rapidly arrested within 2.5 min by NaF (Fig. 4). In the presence of cycloheximide the acetylation of ribosomal proteins and protein synthesis continued, although at a much reduced level as compared to the control in Fig. 3. ^{3}H and ^{14}C radioactivity was found associated with the ribosomes obtained from the reticulocytes after 10 min of incubation with [^{3}H]acetate and [^{14}C]aminoacid mixture

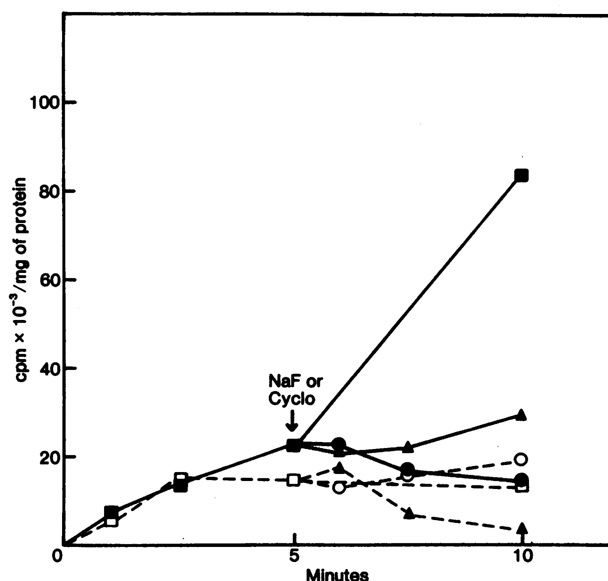

FIG. 3. [^{3}H]Acetate (0.1 mCi/ml) and [^{14}C]aminoacid mixture (1 μCi/ml) were added to a 40-ml reticulocyte suspension (2.3 $\times$ 10^9 cells per ml) and then incubated at 37°. Samples were removed at 1, 2.5, and 5 min (controls). NaF (10 mM) or cycloheximide (*Cyclo*) (0.2 mM) was then added to the suspension. Samples were then removed at 1, 2.5, and 5 min after addition of the inhibitors. Ribosomal proteins and nascent chains were isolated as described in the *text*. ■, Control; effect of (▲) cycloheximide and (●) NaF on incorporation of [^{3}H]acetate into ribosomal proteins. □, Control; effect of (△) cycloheximide and (○) NaF on incorporation of [^{14}C]aminoacids into nascent chains.

(Fig. 5). However, in the presence of NaF or cycloheximide the polyribosomes were rapidly broken down and there was minimal incorporation of isotopes into ribosomes greater than dimers. Significant radioactivity was found in the region of monomers and subunits.

To eliminate the possibility that the effect of NaF or cycloheximide observed on the ribosomal acetylation might be due to an inhibition of the activity of acetyltransferase, we determined the effect of these inhibitors on the activity of the enzyme. As shown in Table 2, NaF or cycloheximide did not inhibit the enzyme activity.

TABLE 1. *Specific activities of some fractionated ribosomal proteins*

Band no.	Specific activity (^{3}H cpm/$A_{570\ nm}$)
7–8	1778
10–11	2196
13–14	890
15–16	1080
17–18	1097
19	780

After gel electrophoresis, the ribosomal proteins were stained with Amido Black and the distinct bands were identified numerically as shown in Fig. 2. The stained protein bands were sliced. The dye was eluted with 1.5 ml of dimethylsulfoxide at 50° for over 2 days and absorbance was measured at 570 nm. The gels were then dissolved in 0.7 ml of hydrogen peroxide at 50° overnight. Both the eluted dye solution and the dissolved gel were mixed with 10 ml of Aquasol scintillator; radioactivity was determined in a liquid scintillation counter.

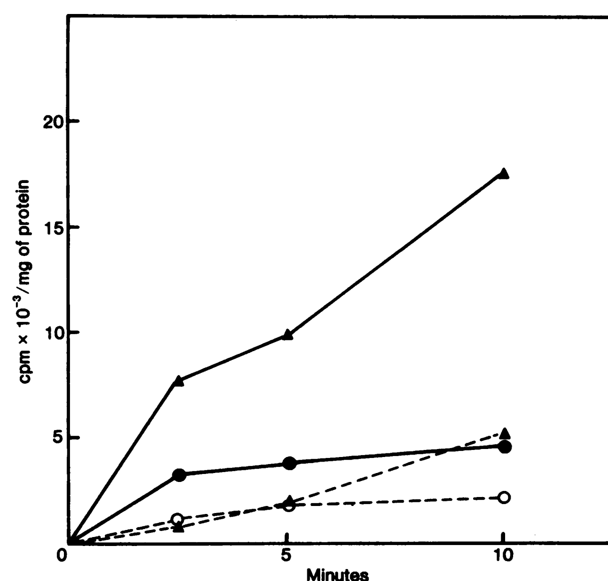

FIG. 4. The reticulocyte suspension was incubated with NaF or cycloheximide for 5 min before addition of [^{3}H]acetate and [^{14}C]aminoacid mixture as described in *legend* of Fig. 3. Samples were removed at 2.5, 5, and 10 min thereafter. Effect of (▲) cycloheximide and (●) NaF on incorporation of [^{3}H]acetate into the ribosomal proteins and of (△) cycloheximide and (○) NaF on ncorporation of [^{14}C]aminoacids into nascent chains.

DISCUSSION

We have shown that the incorporation of [^{3}H]acetate into reticulocyte ribosomal proteins occurred within 10 min. Several prominent bands of the ribosomal proteins separated by polyacrylamide–urea gel electrophoresis were highly acetylated (Table 1). The ^{3}H radioactivity was released by acid hydrolysis. These findings were consistent with our previous report on the acetylation of ribosomal proteins in rat tissues (6).

We have presented evidence that the acetylation of ribosomal proteins is closely associated with protein synthesis in reticulocytes. Inhibition of protein synthesis was accompanied by reduced acetylation of ribosomal proteins. We suggested

TABLE 2. *Effect of NaF and cycloheximide on the activity of acetyltransferase*

	Nuclear acetyltransferase	Cytoplasmic acetyltransferase
Complete system	22,529	412
+NaF		
10 mM	21,353	390
30 mM	20,588	415
+Cycloheximide		
10 mM	22,529	428
30 mM	20,179	423

The activity of cytoplasmic acetyltransferase from reticulocytes and nuclear acetyltransferase from rat liver was assayed as described (6, 15). Ribosomes isolated from reticulocyte were used as substrate. The activity of the enzyme was expressed as cpm/mg of protein.

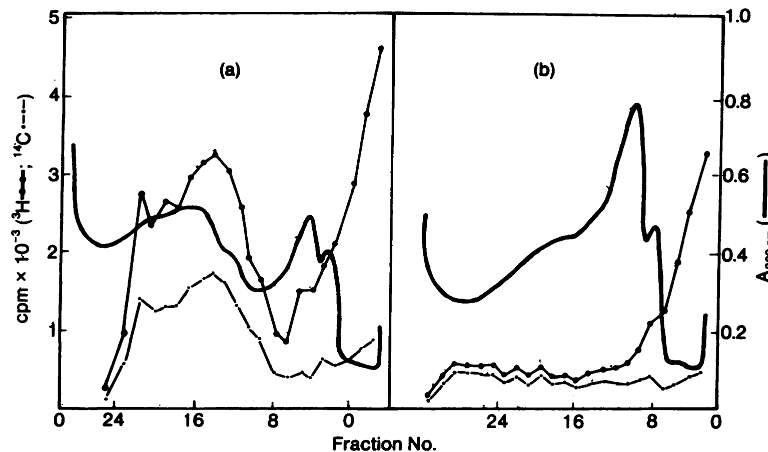

FIG. 5. The reticulocytes were first incubated with or without NaF (10 mM) for 10 min at 37°; [^{3}H]acetate and [^{14}C]aminoacids were then added to the cell suspensions. Ten minutes later, ribosomes from the control (*a*) and NaF-treated reticulocytes (*b*) were isolated from the lysate. Radioactivity distribution in the ribosomes was analyzed on a 10–30% sucrose density gradient by centrifugation at 178,000 × *g* for 30 min. The results with cycloheximide-treated reticulocyte ribosomes were similar to those of NaF-treated ones.

previously (9) that acetylation of ribosomal proteins may play an important role in the early steps of protein synthesis. We previously observed (16) that acetylation of ribosomal proteins was stimulated in regenerating liver and was closely associated with an increase in protein synthesis during this period. We also found that some specific ribosomal proteins were highly acetylated during 6 and 14 hr after partial hepatectomy.

NaF inhibits the early events of initiation (17) but has a very limited effect on the elongation process (18). We found that acetylation of ribosomal proteins was greatly reduced after incubation with NaF (Fig. 3). In the presence of cycloheximide, although the acetylation of ribosomal proteins was reduced, the uptake of [^{3}H]acetate into ribosomal proteins was significantly increased with time (Fig. 4). This observation is consistent with the findings of Balkow *et al.* (19), that a slow regeneration of the initiation complex was demonstrated under conditions of blocked translation by cycloheximide. In addition, we observed previously (6) that measurement of the specific radioactivity of acetylated ribosomal proteins showed a higher incorporation of acetate into small subunits than into large subunits. Thus, our results suggest that acetylation of ribosomal proteins may occur at a time when the initiation complex is being formed.

This work was supported by the Medical Research Council of Canada and the Ontario Heart Foundation. The highly competent assistance of Mrs. K. Yao is acknowledged.

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