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Sites of Biological Methylation of Proteins in Cultured Chick Muscle Cells†

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ABSTRACT: The methylation of myosin and other proteins has been studied using primary cultures of 12-day-old embryonic chick leg muscle. The methyl group of [*Me*-^{3}H]methionine is incorporated into basic amino acid residues with the formation of N^{ϵ}-monomethyllysine, N^{ϵ}-dimethyllysine, N^{ϵ}-trimethyllysine, 3-methylhistidine, N^{G}-monomethylarginine, and N^{G}-dimethylarginine which are isolated from acid hydrolysates of purified myosin, and of proteins from polysomes and from the cytosol of the cultured muscle cells. In the presence of 0.1 m*M* cycloheximide, incorporation of [*Me*-^{3}H]methionine into the polysome-bound proteins was decreased to 16.3% of control levels with no change in the pattern of incorporation into the basic amino acid residues, although protein synthesis was inhibited 97.5%. When protein synthesis was allowed to resume in such cultures by the removal of cycloheximide, polypeptides containing labeled N-methylated residues were released from polysomes into the soluble fraction. Polypeptides containing N-methylated amino acids were also released from polysomes following treatment with 2 m*M* puromycin. Peptidyl-tRNA, isolated from ribosomes after exposure of cultures to [*Me*-^{3}H]methionine, contained labeled N-methylated amino acids. When [*Me*-^{3}H]methionine was incorporated in the presence of cycloheximide, the isolated peptidyl-tRNA still contained N-methylated amino acids although the amount of methylation was 22.4% of control levels. These experiments demonstrate that N-methylation of basic amino acid residues in proteins may occur while the polypeptide is still being synthesized on the ribosome. In addition, N-methylation can occur on the nascent polypeptides in the absence of protein synthesis.

Basic amino acids which are modified by N-methylation are present in proteins from many sources (Paik and Kim, 1971). Among the sources which have been investigated are the histones (Allfrey, 1971), ribosomal proteins (Comb et al., 1966; Terhorst et al., 1972; Reporter, 1973a; Alix and Hayes, 1974; Chang and Chang, 1974; Chang et al., 1974), plant and fungal cytochrome *c* (Lemberg and Barrett, 1973), rhodopsin (Reporter and Reed, 1972), and skeletal muscle actin and myosin from rabbit (Johnson and Perry, 1970), chicken (Krzysik et al., 1971), bovine (Asatoor and Armstrong, 1967), and cat (Kuehl and Adelstein, 1970), as well as cultured muscle from rat (Reporter, 1969) and chicken (Morse and McManus, 1973).

N-Methylation of arginine, lysine, and histidine occurs after incorporation of the unmodified amino acids into protein (Paik and Kim, 1971). This post-translational modification of amino acids is catalyzed by methyltransferase(s) which use *S*-adenosylmethionine as the methyl donor (Paik and Kim, 1971; Hardy et al., 1970; Krzysik et al., 1971; Reporter, 1973b). Attempts to find aminoacyl-tRNAs charged with N-methylated amino acids have been unsuccessful (Kim and Paik, 1965; Young et al., 1970, 1972; Reporter, 1973b).

This paper is concerned with the question of whether the post-translational methylation of amino acids takes place while the polypeptide is still being synthesized on the ribo-

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some or after termination and release of an unmodified protein from the ribosome. A preliminary account of the results of a study of this problem in cultured chick embryonic skeletal muscle cells was presented (Morse and McManus, 1973) and concomitant with this work, Reporter (1973b) described experiments using rat muscle cultures in which he obtained indirect evidence for the N-methylation of basic amino acid residues in support of the methylation of nascent polypeptide chains. The present paper describes the pattern of methyl group modification of free proteins and polysome-bound polypeptides in cultured chick muscle cells and includes direct analysis of methylated amino acids in peptidyl-tRNAs derived from ribosomes of cultured muscle.

Experimental Section

Materials

[*Me*-^{3}H]-L-Methionine, 0.5–2 Ci/mmol, was obtained from ICN Isotope and Nuclear Division. ECTEOLA-cellulose (fine mesh), puromycin dihydrochloride, cycloheximide, bovine pancreatic ribonuclease (type 1-A, salt and protease-free), trypsin, type III, and His(3-Me) were purchased from the Sigma Chemical Co. Ribonuclease-free sucrose and ultrapure urea were purchased from Schwarz/Mann Biochemicals. Horse serum, nutrient mixture F-10, penicillin G, and streptomycin sulfate were obtained from Grand Island Biologicals. Arg(G-Me),[1] N^G,$N^{G'}$-dimethyl-L-arginine, N^G-dimethyl-L-arginine, N^ϵ-trimethyl-L-lysine-bis(*p*-hydroxyazobenzene-*p*-sulfonate)·2H_2O, and soybean trypsin inhibitor were obtained from Calbiochem. Lys(Me_2) and N^α-methyl-L-lysine were purchased from the Cyclo Chemical Corp.

Methods

A. Muscle Cell Culture. Muscle cell cultures were prepared from the leg muscles of 12-day-old embryos of white leghorn chickens using the method of Coleman and Coleman (1968). Cells were plated at an initial density of 5 × 10^6 cells/150-mm dish on collagen coated Falcon plastic petri dishes with 20 ml of medium or 8 × 10^5 cells on 60-mm dishes in 4 ml of medium. Medium was changed on days 2, 4, 5, and 6, and the cultures were used on the seventh day.

B. Isolation of Myosin from Cultured Muscle Cells and Preparation of HMM. Myosin was isolated from 7-day-old muscle cultures by a modification of the isolation procedure of Paterson and Strohman (1972). The cells were lysed with 0.5% Triton X-100 as described previously (Morse et al., 1971). One volume of 0.94 *M* KCl–0.02 *M* KH_2PO_4–0.02 *M* sodium pyrophosphate was added to the cell lysate and the suspension was stirred for 30 min in an ice bath and then centrifuged for 10 min at 17,300*g*. The supernatant solution was removed and 25 mg of unlabeled purified chick myosin was added and the solution was then diluted by adding it slowly with stirring to 12 volumes of cold glass-distilled water. The resulting white precipitate was collected by centrifugation and the crude myosin was put through four cycles of solution and precipitation. The final precipitate was stored in 50% glycerol at −20° if it was not used immediately. ATPase activity was assayed as described by Perry (1955).

HMM was prepared from myosin by the procedure of Lowey et al. (1969). Myosin and HMM were checked for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described by Dow and Stracher (1971). The heavy chains of myosin were recovered from ten disc gel columns, previously loaded with 100 μg of protein and electrophoresed, by cutting out the region containing the heavy chains, sectioning this into 1-mm slices, and homogenizing the slices with 2 volumes of 5% sodium dodecyl sulfate–0.1 *M* NaCl in an Omnimixer. After letting this mixture stand overnight, the gel mixture was recovered by centrifugation and the extraction was repeated. About 90% of the protein was obtained in the pooled extracts.

Protein was measured by the Lowry (Lowry et al., 1951) or the biuret methods (Gornall et al., 1949).

C. Preparation and Sucrose Gradient Analysis of Polysomes. Extraction and sucrose density gradient resolution and analysis of polysomes have been described previously (Morse et al., 1971) except that a Spinco SW-27 rotor was used. The polysome extracts from 7-day-old cultures were layered on to 38.4 ml of 15–40% linear sucrose gradients prepared in KMT buffer and centrifuged at 27,000 rpm (131,000 g) for 2 hr at 4° without braking.

D. Isolation of Peptidyl-tRNAs from the Ribosome Fraction. Cultured cells were scraped from 150-mm petri dishes using 20 ml of KMT buffer per plate and centrifuged at 200 *g* for 10 min. The cell pellet was resuspended in 4 ml of NMP buffer and the cells were lysed by triturating in 0.5% Triton X-100 as described by Morse et al. (1971). Ribosomes were prepared from the cell lysate and dissociated in 1.1 ml of a solution containing 0.5% (w/v) sodium dodecyl sulfate, 0.1 *M* NaCl, 0.1 *M* sodium formate–formic acid buffer at pH 4.7, 1% (w/v) BRIJ-35, and 6 *M* urea. After centrifuging to remove any undissolved particulates, this mixture was chromatographed, as described below, using methods that are described more fully by Cioli and Lennox (1973a). The dissociated ribosomes were diluted to a final volume of 10 ml with the same buffer solution except that sodium dodecyl sulfate was omitted. This diluted sample was applied to an 0.6 × 7.5 cm column of washed ECTEOLA-cellulose (0.3 mequiv/g) which was equilibrated with the starting buffer. The void volume was designated as fraction 1 and an additional two 10-ml fractions (fractions 2 and 3) of starting buffer eluates were collected. The column was then developed by increasing the NaCl concentration to 1.0 *M* in the same buffer; 30 ml of eluting buffer was passed through the column and collected in 10-ml portions designated as fractions 4, 5, and 6. Cioli and Lennox (1973a,b) have shown that the fractions eluted with 1.0 *M* NaCl contain peptidyl-tRNAs and our results confirm this. Samples of these fractions were combined with 2 mg of carrier bovine serum albumin, 1 volume of 20% trichloroacetic acid was added, and the precipitated proteins were washed as described below and prepared for radioactive assay. The remainder was used for rechromatography and for further verification of the identities of the fractions.

E. Amino Acid Analysis of Proteins. Methylated basic amino acids were analyzed by a modification of the methods of Krzysik et al. (1971) and Deibler and Martenson (1973). The samples were hydrolyzed in 6 *N* HCl in nitrogen flushed, sealed glass ampoules for 48–72 hr at 110°. To

[1] Abbreviations used are: Arg(G-Me), N^G-monomethylarginine; Arg(G-Me_2), N^G-dimethylarginine and N^G,$N^{G'}$-dimethylarginine; BRIJ-35, poly(oxyethylene)-23-lauryl ether; HMM, heavy meromyosin; His(3-Me), *N*-3-methylhistidine; KMT buffer, 0.25 *M* KCl–0.01 *M* $MgCl_2$–0.01 *M* Tris-HCl buffer (pH 7.4); Lys(Me), N^ϵ-monomethyllysine; Lys(Me_2), N^ϵ-dimethyllysine; Lys(Me_3), N^ϵ-trimethyllysine; NMP buffer, 0.25 *M* NaCl–0.01 *M* $MgCl_2$–0.025 *M* sodium phosphate buffer (pH 6.7).

Table I: Distribution of Radioactivity from Incorporation of [*Me*-^{3}H] Methionine into Methylated Basic Amino Acid Residues in Cultured Muscle Cells.

Expt No.	Protein Analyzed	% Total Radioactivity in Basic Amino Acids					
		Lys(Me)	Lys(Me_2)	Lys(Me_3)	His(3-Me)	Arg(G-Me_2)	Arg(G-Me)
1[a]	Total[c]	3.0	10.1	29.4	8.4	46.5	2.6
	Myosin	2.9	8.1	38.5	10.5	39.2	1.0
2[b]	Myosin	2.0	4.0	41.7	10.8	40.3	1.2
	HMM	1.9	4.2	56.0	4.1	32.0	1.7

[a] Seven 150-mm plates of 7-day-old muscle cultures were labeled with 25 μCi of [*Me*-^{3}H] methionine/plate and incubated for 20 hr. Basic amino acid residues from the acid hydrolysate of total protein from one plate contained 146,662 cpm (4.5% of activity of hydrolysate). Basic amino acid residues from the acid hydrolysate of 2 mg of myosin contained 23,098 cpm. [b] Eight 150-mm plates of 7-day-old muscle cultures were labeled with 25 μCi of [*Me*-^{3}H] methionine/plate and incubated for 18 hr. Basic methylated amino acid residues from the acid hydrolysate of 1.6 mg of myosin contained 16,614 cpm, representing 4.2% of activity of the total hydrolysate. HMM was prepared from labeled myosin. The acid hydrolysate from 9.5 mg of HMM was analyzed. Basic methylated amino acid residues contained 7773 cpm, representing 2.5% of activity of the total hydrolysate. [c] For analysis of total protein, cells were collected, washed with KMT buffer containing 1 m*M* methionine, and lysed with 0.5% Triton X-100 and the protein was precipitated with 10% trichloroacetic acid, washed, and hydrolyzed. Method A was used for analysis of methylated amino acid residues.

serve as markers for the analysis, 0.1 m*M* (0.2 ml of 0.5 μmol/ml 0.01 *N* HCl) of α-monomethyl-L-lysine, Lys(Me), His(3-Me), Lys(Me_2), Lys(Me_3), the N^G,$N^{G'}$ isomer of Arg(G-Me_2), and Arg(G-Me) were added to the samples. Free Lys(Me_3) was generated from N^ϵ-trimethyl-L-lysine-bis(*p*-hydroxyazobenzene-*p*-sulfonate-H_2O by application of the derivative to a Dowex-1-acetate column. The free acid is eluted as the acetate salt. For calibration of columns, 0.02 μmol of each of these amino acids were used.

The sample in 2 ml of water was chromatographed on a 0.9 × 45 cm column of Beckman UR-30 resin which was preequilibrated with 0.35 *N* sodium citrate buffer (pH 5.84). The column was developed with this buffer using a flow rate of 46 ml/hr at 28°. At 340 min the temperature was increased to 55°, NaCl was added to give 1.18 *N* Na^+, and the pH was adjusted to 7.50. The run was stopped after 450 min (method A).

This procedure was modified (method B) by preequilibrating and starting development with 0.545 *N* sodium citrate at pH 5.84. At 220 min the temperature was increased to 55°, NaCl was added to give 1.345 *N* Na^+, and the pH was adjusted to 7.50. The run was stopped at 340 min. One-fifth of the effluent was analyzed for amino acid concentration and 4/5 was collected in fractions at 2-min intervals for counting as described below.

F. Analysis of Radioactive Samples. Radioactivity in polysome fractions obtained following density gradient centrifugation and fractions collected during amino acid analysis of protein hydrolysates were measured by scintillation counting using a Triton X-100–toluene counting mixture and procedure as described previously by Krzysik et al. (1971). The counting efficiency for ^{3}H was 37.5%. For radioactive analysis of proteins, 1 volume of 20% (w/v) trichloroacetic acid was added to the sample along with 5 mg of bovine serum albumin. The precipitate was centrifuged at 1000*g*, treated for 20 min with 10% trichloroacetic acid at 90°, and then washed, either by centrifugation, or on Whatman GF/A glass fiber filters, with 10% trichloroacetic acid, 95% ethanol, ethanol–ether–chloroform (2:2:1, v/v), and ether (Allfrey et al., 1957). The washed and dried precipitates were dissolved in 0.2 ml of NCS solubilizer (Amersham-Searle) by incubating at 50° for 1 hr and then mixed with 15 ml of 4.3% (v/v) Liquifluor–toluene solution (New England Nuclear). The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 3375.

Results

Incorporation of [Me-^{3}H]Methionine into Proteins of Cultured Muscle Cells. Preliminary experiments were performed to provide some background for examining the process of methylation of the proteins of cultured chick muscle cells. A representative 7-day culture was treated with May-Grünwald and Giemsa stains and nuclei were counted in 50 randomly selected fields at 400× magnification. The nuclei in myotubes represented 72.8% of the population of 1519 nuclei counted, with a 95% confidence interval of ±3.8%. Thus we are looking mainly at the methylation of contractile proteins of muscle, though contractile proteins in nonmyogenic cells (Rubinstein et al., 1974) may also be methylated.

Seven-day muscle cultures were incubated for 15 min to 24 hr with 5 μCi of [*Me*-^{3}H]methionine (143 Ci/mol) per plate and labeled myosin was isolated and purified after addition of carrier myosin. Incorporation was linear over the first 4 hr. According to Coleman and Coleman (1968), the rate of myosin synthesis is maximal at this stage of culture under the conditions used here and myosin accounts for about 1.6% of the total protein, or about 250 μg of myosin/150-mm plate. Table I shows the incorporation of [^{3}H]methyl groups from [*Me*-^{3}H]methionine into methylated basic amino acid residues of total protein and of myosin purified from the 7-day-old cultures. Results are expressed in terms of percent of radioactivity in total protein and myosin recovered in the total basic amino acid fraction and the actual cpm in each fraction are given in the legend to Table I. As is seen in experiment 1 of Table I a total of between 75.9 and 77.7% of the ^{3}H derived from [*Me*-^{3}H]methionine is found in Lys(Me_3) and Arg(G-Me_2) in total protein and in myosin. Appreciable amounts of Lys(Me_2) are recovered from the myosin hydrolysate, although as is seen in experiment 2, the contribution of this amino acid to the total radioactivity is variable. That these methylated amino acids are associated with myosin is supported by the results shown in experiment 2 in which HMM was prepared from myosin. His(3-Me) and the N^ϵ-methylated lysine residues are localized in the head protein of myosin (Johnson et al., 1967; Kuehl and Adelstein, 1969) and comparison of the distribution of radioactivity among the methylated amino acids obtained from myosin and HMM hydrolysates shows little difference in the radioactive pattern except for a lower radioactivity in His(3-Me), consistent with the presence of some actin in the myosin prepara-

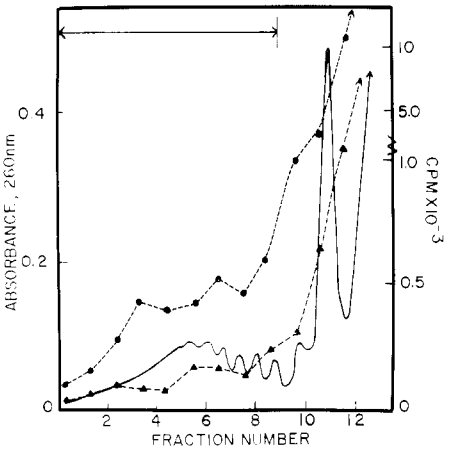

FIGURE 1: Effect of cycloheximide on the incorporation of [*Me*-^{3}H]methionine into muscle polysomes. Cycloheximide was added to three 150-mm plates of 7-day-old muscle cultures to give a final concentration of 0.1 m*M*. After 5 min, 50 μCi of [*Me*-^{3}H]methionine was added to each of the three plates containing cycloheximide and to one additional control plate which had not been treated with cycloheximide. After incubation for 4 hr, the cells were collected and the polysomes were isolated and resolved in 15–40% sucrose gradients as described in Methods. The control sample was centrifuged on one gradient and the cycloheximide-treated sample was divided and run on three gradients. Samples of 0.5 ml were taken for radioactive counting from each of the 14 fractions of the control gradient (●) and from one of the three cycloheximide gradients (▲). The profile of absorbance at 260 nm was taken from the cycloheximide treated sample which was counted. The absorbance profiles of the control gradient and the other two cycloheximide treated gradients were nearly identical with this one. The horizontal line indicates the polysome region of the gradient which was combined and subjected to amino acid analysis (see Table II).

tion. In fact, a small amount of contaminating actin was found when an aliquot of labeled myosin was run in sodium dodecyl sulfate gel electrophoresis (Dow and Stracher, 1971). Both Arg(G-Me_2) and Arg(G-Me) are found in the HMM portion of myosin. In a separate experiment, the heavy chains of labeled myosin were recovered from disc gels after sodium dodecyl sulfate acrylamide gel electrophoresis and analysis of this protein showed that 58% of the radioactivity of the basic amino acid fraction was recovered in Lys(Me_3) and 22.6% in Arg(G-Me_2). The lack of major differences in the pattern of the labeled methylated amino acids in myosin as compared to the total protein from the cell lysate suggest that the major methylated protein of the muscle is myosin. The composition of methylated residues in myosin from these cultured chick leg muscles is in qualitative agreement with other analyses of embryonic skeletal muscle myosin (Kuehl and Adelstein, 1970; Reporter and Corbin, 1971).

Effect of Cycloheximide on Distribution of Methylated Residues in Muscle Cultures. The above experiments demonstrate that protein methylation involving histidine, lysine, and arginine residues of myosin and other proteins has occurred in the cultured muscle cells but no information existed in these experiments as to whether the process of methylation of these residues was in any way dependent on concomitant protein synthesis other than in providing new peptides as substrates for the methylation process. Therefore, it was of interest to study methylation under conditions where protein synthesis was inhibited. Cycloheximide is well established as an inhibitor of amino acid incorporation into proteins on cytoplasmic ribosomes of eukaryotes and the polysomes appear to be both physiologically and morphologically frozen by cycloheximide (Pestka, 1971).

Table II: Incorporation of [*Me*-^{3}H]Methionine into Methylated Basic Amino Acid Residues in the Presence of Cycloheximide.[a]

Fraction Obtained from Sucrose Density Gradient	Control (cpm)	Cycloheximide-Treated cpm	Cycloheximide-Treated % of Control
Polysomes	3,114	507	16.3
Non-polysomes	6,518	8,062	123.7
Total sample on gradient	9,633	8,569	88.9
Total cytoplasmic protein[b]	1,992,450	446,620	22.4

[a] The material remaining after aliquots were taken for radioactive counting of the sucrose gradient (shown in Figure 1) was combined into polysome fractions (indicated by arrow in Figure 1: fractions 1–10) and non-polysome fractions (fractions 11–14). The protein was precipitated from the combined fractions with 10% trichloroacetic acid and washed, and the precipitates were hydrolyzed and analyzed for basic amino acid content as described in Methods. The counts indicate the total radioactivity in the methylated amino acid peaks after amino acid analysis. The radioactivity from the control plate was multiplied by 3 and both sets were normalized to take into account the different proportions of the samples removed previously for the radioactive profile shown in Figure 1. [b] The polysome extract of a 12,000*g* supernatant from the cell lysate was used for assessment of total radioactivity incorporated into cytoplasmic protein. The protein was washed with 10% trichloroacetic acid and lipid solvents as described in the Methods before measurement of radioactivity.

Figure 1 illustrates the results of an experiment in which three 7-day-old muscle cultures were preincubated with 0.1 m*M* cycloheximide and, after 5 min, 50 μCi of [*Me*-^{3}H]methionine was added to each of the cultures as well as to untreated control cultures. Preliminary experiments showed that protein synthesis on cytoplasmic ribosomes is inhibited 97.5% by 0.1 m*M* cycloheximide. Four hours later, the cells were collected, and polysomes were isolated, resolved in 15–40% sucrose gradients, and analyzed for tritium as described in the Methods. The polysome profile, as shown by the absorbance at 260 nm, is characteristic of developing muscle cultures (Morse et al., 1971) and both the polysome and non-polysomal regions of the sucrose gradients are labeled significantly with lower incorporation of radioactivity into the cycloheximide-treated sample. The polysome region, indicated by the solid horizontal line in Figure 1 and encompassing fractions 1–10, was combined, and the remaining fractions were pooled and designated as the non-polysome region. Proteins were precipitated from these pooled fractions and analyzed for labeled methylated amino acid residues as shown in Table II. Incorporation of [*Me*-^{3}H]methionine into total methylated amino acids associated with the polysome fraction was decreased to 16.3% of the control level by cycloheximide, an amount still indicative of some methylation of amino acids, independent of protein synthesis, in this fraction. The cycloheximide-treated non-polysome fraction had 23.7% more radioactivity associated with the methylated amino acids than did the control, totaling 8062 cpm, as contrasted to 6518 cpm in the control. This indicates that cycloheximide does not inhibit methylation of the amino acid residues. Figure 2 shows a similar experiment in which twice as many cells were used as in the previously described experiment and 25 fractions were collected from the 15–40% sucrose gradient in an effort to improve resolution of the radioactive profile of the polysomes. The polysome-containing regions, consisting of fractions 1–21, were pooled; proteins were recovered and analyzed for methylated basic amino acid residues. The hy-

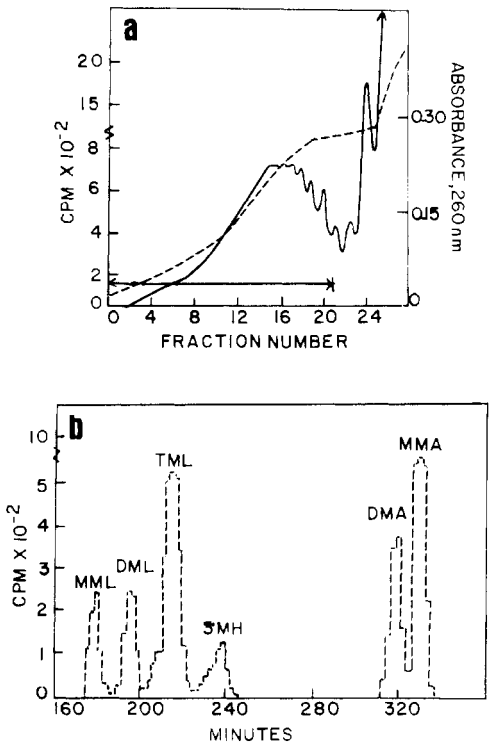

FIGURE 2: Incorporation of [*Me*-^{3}H]methionine into methylated basic amino acids of the polysomes of cycloheximide-treated cells. Six 150-mm plates of 7-day-old cultures were preincubated for 5 min with 0.1 m*M* cycloheximide followed by addition of 50 μCi of [*Me*-^{3}H]methionine/culture. Following a 4-hr incubation, polysomes were isolated and analyzed for incorporation of ^{3}H. (a) Polysome profile from 15 to 40% linear sucrose gradient showing absorbance at 260 nm (—) and incorporation of ^{3}H into resolved fractions (- - -). The horizontal arrow indicates fractions pooled for acid hydrolysis. (b) Profile of ^{3}H incorporation into methylated basic amino acid residues obtained by ion exchange chromatography of acid hydrolysate of contents of tubes 1–21 in (a).

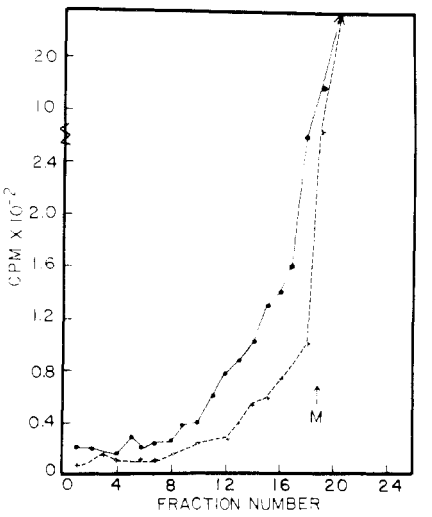

FIGURE 3: [*Me*-^{3}H]Methionine and cycloheximide pulse/chase. The cells from each plate were extracted separately in 1.0 ml of KMT buffer with 0.5% Triton X-100 and the 12,000*g* supernatant was layered onto a 12.4-ml linear gradient of 15–40% sucrose. The gradients were centrifuged in a Spinco SW-40 rotor at 30,000 rpm (98,800*g*) for 45 min at 4° with the brake on. Fractions of 0.5 ml were collected and analyzed for radioactivity. (—) Sample which was not chased; (- - -) sample which was chased for 30 min. The position of the monomers is indicated by th letter "M" in the figure.

drolysate contained a total of 51,060 cpm and the profile of the radioactive methylated amino acids in the hydrolysate is shown in Figure 2b. Lys(Me_3), Arg(G-Me), and Arg(G-Me_2) account for 78.4% of the total radioactivity in the basic residues. A higher percentage of Arg(G-Me) is apparent here than is found in purified myosin, a not unexpected result since other methylated proteins, e.g., histones and ribosomal proteins, are in the polysome region of the sucrose gradients.

If the methylation observed in the polysomes in the presence of cycloheximide is indeed occurring on nascent polypeptide chains, then some of the amino acid residues which are methylated on the polysomes should be released when protein synthesis is restored to normal by removal of cycloheximide. Colombo et al. (1966) have shown that inhibition of protein synthesis by cycloheximide in intact reticulocytes is reversible. In the following experiment, protein synthesis was first inhibited with cycloheximide and then [*Me*-^{3}H]methionine was added for a 1-hr pulse. In the chase, cycloheximide was removed and the specific activity of the labeled methionine was reduced by rinsing the cells with methionine-supplemented medium. Thus, during the pulse, with protein synthesis inhibited, methylation should account for most of the incorporated radioactivity. During the chase, protein synthesis resumes but the incorporation of labeled methionine into protein should be very low because the specific activity of the methionine has been much reduced. Preliminary experiments showed that, in order to restore the rate of protein synthesis in the muscle cultures to normal levels after cycloheximide inhibition, it was necessary to wash the cells with culture medium at least ten times. Having established appropriate conditions for a pulse-chase experiment, six 7-day-old cultures on 60-mm plates were preincubated for 5 min with 0.1 m*M* cycloheximide and then each was pulsed for 1 hr with 100 μCi of [*Me*-^{3}H]methionine. Labeled methionine and cycloheximide were removed by washing the cells ten times with 5 ml of culture medium supplemented with 1 m*M* L-methionine. The cells from two plates were collected immediately while the remaining cultures were allowed to resume protein synthesis for 30, 45, 60, and 75 min, in the presence of normal medium supplemented with 1 m*M* methionine. The cells were then chilled quickly by rinsing with cold KMT buffer supplemented with 2 m*M* L-methionine and collected. Polysome extracts were prepared, layered on sucrose gradients, and centrifuged. The results of this experiment are shown in Figure 3. Only the results of the 30-min chase are shown since similar results were obtained after 45, 60, and 75 min. When protein synthesis resumed after cessation of cycloheximide inhibition, 55% of the incorporated radioactivity was eliminated from the polysome region as the polypeptide chains were completed and released during the chase period. The level of radioactivity in the polysome area was not adequate to permit analysis of the methylated amino acid residues, but some evidence for the release of methylated residues from the polysome region was afforded by a separate companion experiment in which soluble proteins in fractions 20–24 were analyzed for radioactive methylated basic amino acid residues before and after a 30-min chase following incubation with cycloheximide and 50 μCi of [*Me*-^{3}H]methionine for 10 min. A total of 4355 cpm were recovered in the methylated residues before the chase, which increased to 5984 cpm after the chase. The distribution of radioactive residues was similar in both samples with 39.6% of the total radioactivity located in Arg(G-Me_2) before the chase, and 44.1% of the total in this residue fol-

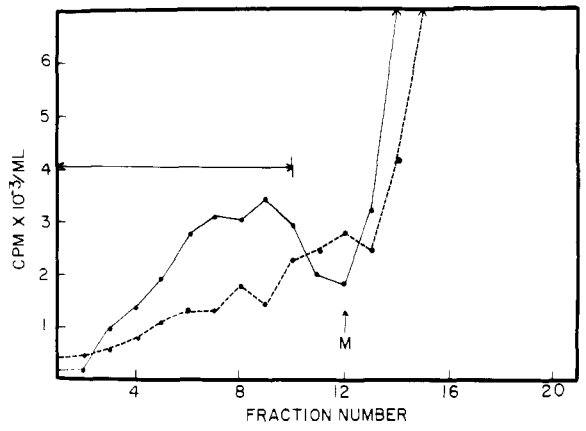

FIGURE 4: The release of methylated proteins from polysomes by puromycin. After a 30-min incubation with a total of 1.5 mCi of [*Me*-^{3}H]methionine in ten 150-mm plates the cultured muscle cells were collected and the polysomes were extracted. Puromycin (20 m*M* in 0.25 ml of KMT buffer) was added to one 2.3-ml aliquot of the supernatant. For the control, 0.25 ml of buffer was added to the other 2.3-ml aliquot. Both aliquots were kept on ice until they were layered onto two sucrose gradients and centrifuged in the SW-27 rotor at 26,000 rpm as described in Methods. The centrifugation was started 45 min after the puromycin was added. The polysome gradients were analyzed for absorbance at 260 nm and collected in 2.2-ml fractions. After removing 0.2 ml from each fraction for radioactive counting, fractions 1–10 (horizontal arrow) were pooled for amino acid analysis (see Table III). (—) Control sample; (- - -) the puromycin-treated sample. The position of the monomers is indicated by the letter "M" in the figure.

Table III: ECTEOLA-Cellulose Chromatography of Ribosome Proteins.[a]

Fraction No.	NaCl Concn	A Ribosomes (cpm)	Rechromatographed Fraction 4 from A[b] B RNase Treated (cpm)	C Untreated (cpm)
Input		428,280	2,908	2,908
1	0.1	61,607	556	24
2	0.1	6,115	124	26
3	0.1	758	26	14
4	1.0	29,080	97	624
5	1.0	1,905	54	93
6	1.0	820		
Total recovered radioactivity		100,285	857	781

[a] One 150-mm plate containing a 7-day-old muscle culture was incubated with 200 μCi of [*Me*-^{3}H] methionine for 95 min. The isolation and chromatography of the ribosomal proteins are described in the Methods. The dimensions of the ECTEOLA-cellulose column were 0.6 × 7.5 cm. [b] Two milliliters from fraction 4 in column A were divided into two 1-ml aliquots. Ribonuclease (100 g) was added in 0.1 ml of starting buffer to one aliquot (B) and 0.1 ml of starting buffer without ribonuclease was added to the other aliquot (C). Both aliquots were incubated at 37° for 30 min and then diluted 1:10 by adding 9 ml of 0.1 *M* formate buffer containing 6 *M* urea and 0.1% (w/v) BRIJ-35 to bring final concentration of NaCl to 0.1 *M*. The diluted samples were then run on new ECTEOLA-cellulose columns.

lowing the chase. The remaining radioactivity was recovered principally in Lys(Me), Lys(Me_2), and Arg(G-Me).

Effect of Puromycin on Protein Methylation. Since puromycin interrupts peptide chain elongation and so releases nascent peptide chains (Morris et al., 1963; Nathans, 1964), this also provides a tool for studying the methylation of growing polypeptide chains on the polysomes in the course of protein synthesis and for examining the site of methylation of basic amino acid residues. Following incubation of ten 7-day-old muscle cultures in 150-mm plates with a total of 1.5 mCi of [*Me*-^{3}H]methionine for 30 min, a cell lysate was prepared and the polysome containing fraction was divided into two parts. One part was incubated with 2 m*M* puromycin at 0° for 45 min before centrifugation in the 15–40% sucrose gradient and the other part was not treated with puromycin. The 12,000*g* supernatant contains adequate soluble factors for protein synthesis (Morse et al., 1971) and, hence, for demonstrating puromycin activity. Figure 4 shows the radioactive profiles obtained when these two samples were run in sucrose density gradients. The patterns indicate that radioactive material was indeed released from the polysomes by the puromycin treatment and fractions 1–10 of the puromycin-treated material (the region indicated by the horizontal line) contained 1482 cpm in the methylated basic amino acids as compared to 2756 cpm in the control, representing 14.7 and 13.3%, respectively, of the total incorporated radioactivity. Thus, an average of 87.6% of the total radioactivity is due to incorporation of methionine residues into protein, but the methyl moiety of methionine is also incorporated into basic amino acid residues, and a portion of the protein containing methylated residues, equivalent to 1274 cpm, has been shifted from the polysomes by treatment with puromycin. The relative proportion of methylated residues in the polysome region is unaltered by puromycin and the released peptides are probably a random sample of the total population of nascent peptides.

Direct Analysis of the Methylation of Nascent Polypeptide Chains. None of the results obtained in the above experiments demonstrated *directly* the occurrence of methylation of basic amino acid residues at the level of the nascent polypeptides, although the findings suggested that such methylation was occurring independent of active protein synthesis. Isolation and analysis of the degree of methylation of nascent polypeptides would provide more direct evidence and would eliminate the possibility that the other results were due to the presence of completed proteins which remain adsorbed to the ribosomes without being covalently linked to tRNA (Cioli and Lennox, 1973a).

Accordingly, cultured cells were labeled with [*Me*-^{3}H]methionine and isolation of the nascent chains as peptidyl-tRNA complexes from ribosomes was performed using the procedure developed by Cioli and Lennox (1973a) as modified from a method described by Ganoza and Nakamoto (1966). Ribosomes with the attached nascent chains were obtained as Cioli and Lennox described, with modification of the medium to provide a higher ionic strength to prevent precipitation and adsorption of myosin on the ribosomes (Heywood et al., 1967). The peptidyl-tRNAs were then released from the ribosomes by dissolving the ribosomes in 0.5% sodium dodecyl sulfate and 6 *M* urea. This extract was made to a final concentration of 0.1 *M* NaCl and then passed through a column of ECTEOLA-cellulose equilibrated with the same solution. Under these conditions, in addition to peptidyl-tRNAs, nucleic acids and nucleotides are retained and these constituents are eluted by increasing the concentration of NaCl to 1.0 *M*. The results of this type of analysis are shown in Table III, together with details of the experimental protocol. Fractions 1–3 represent 10-ml eluates using 0.1 *M* NaCl as eluting agent, while fractions 4–6 are eluted with 1.0 *M* NaCl. Carrier bovine

Table IV: Radioactivity in Methylated Amino Acids Obtained from Hydrolysates of Eluates Following ECTEOLA-Cellulose Chromatography of Ribosome Fractions.[a]

Fraction No.	NaCl used for Elution (*M*)	cpm in Methylated Basic Amino Acids		
		Control	Cycloheximide treated	% of Control
1 + 2	0.1	60,139	9,553	15.9
5 + 6	1.0	8,922	2,001	22.4

[a] Sixteen 150-mm plates of cultured muscle cells were each incubated with 100 μCi of [*Me*-^{3}H] methionine for 3 hr, with eight of these plates incubated with 0.1 m*M* cycloheximide starting 5 min before addition of labeled methionine. The isolation and chromatography of the ribosome proteins were performed as described in Methods except that an additional 10 ml of both starting buffer (0.1 *M* NaCl) and eluting buffer (1.0 *M* NaCl) were added to the ECTEOLA-cellulose column. Indicated fractions from the experiment were combined and 5 mg of bovine serum albumin was added. The protein was then precipitated with 10% trichloroacetic acid and the precipitate was hydrolyzed and analyzed for methylated amino acid content.

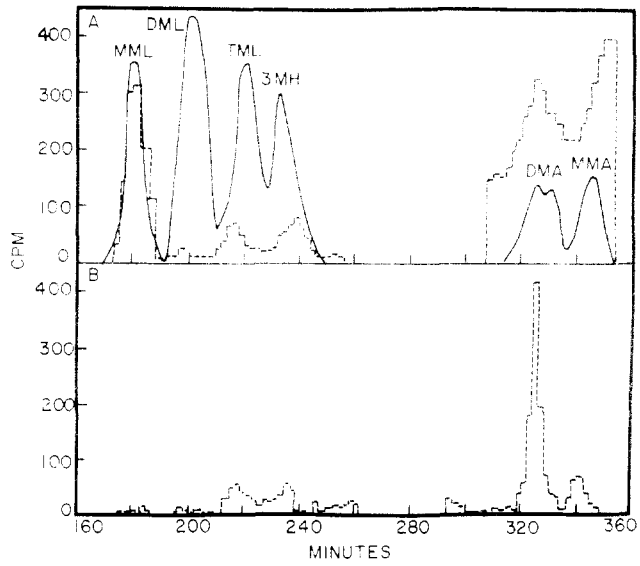

FIGURE 5: Radioactive profiles of methylated basic amino acids from acid hydrolysates of peptidyl-tRNA isolated from cycloheximide-treated and control 7-day-old muscle cultures. See text and legend to Table IV for details of preparation of these samples. (A) Profile of standard methylated basic amino acids (—) and radioactivity (- - -) in peptidyl-tRNA hydrolysate from control cells. (B) Radioactive profile of methylated basic amino acids in peptidyl-tRNA hydrolysate from cycloheximide-treated cells.

serum albumin (2 mg) was added to each of these fractions, followed by 1 volume of 20% trichloroacetic acid. After 20 min, the trichloroacetic acid precipitates were collected by centrifugation, washed, and prepared for radioactive assay as described in the Methods. Of the 428,280 cpm which were applied to the ion exchange column, a total of 68,480 cpm was collected in the 0.1 *M* NaCl eluate and precipitable with trichloroacetic acid, representing protein which did not adhere to the ECTEOLA-cellulose, while 31,805 cpm was eluted in the high ionic strength medium and precipitable with trichloroacetic acid consistent with the presence of labeled peptidyl-tRNA. One-tenth of the fraction 4 eluate was incubated with 100 μg of pancreatic ribonuclease at 37° for 30 min, diluted with 0.1 *M* formate buffer containing 6 *M* urea and 0.1% BRIJ-35 to give the ionic strength of the starting buffer, and applied to a second ECTEOLA-cellulose column. The results of the development of this column are shown in column B of Table III. Ribonuclease treatment shifted 82.4% of the total recovered radioactivity from fraction 4 into the 0.1 *M* NaCl fractions. A similar sample which was not treated with ribonuclease was chromatographed a second time using the same conditions and 91.8% of the total recovered radioactivity was recovered in 1.0 *M* NaCl fractions. This result is seen in column C. When an aliquot of fraction 1 from chromatography of the original sample was incubated with and without ribonuclease treatment and then rechromatographed, 90.9 and 81.5%, respectively, of the total recovered radioactivity remained in the 0.1 *M* NaCl fractions. These results indicate that the ^{3}H from [*Me*-^{3}H]methionine was incorporated into material capable of being retained on the ion exchanger by virtue of the presence of strongly negatively charged groups, which were susceptible to ribonuclease digestion, and precipitable by trichloroacetic acid. These properties are consistent with the presence of peptidyl-tRNA. More thorough proof that the material released from the ECTEOLA-cellulose by a high ionic strength medium is peptidyl-tRNA is provided in the experiments of Cioli and Lennox (1973a,b). The overall recovery of radioactivity, based on the levels of radioactivity of the initial samples applied to the column, was between 23.4 and 29.5%. This low recovery is ascribable to the presence of appreciable amounts of methylated purine and pyrimidine bases in tRNA and rRNA (Kerr and Borek, 1973) and to the trichloroacetic acid precipitation procedure used which would tend to exclude low molecular weight peptides from analysis. In addition, any methylated lipids (Thompson, 1970) would be extracted by the lipid solvents used subsequent to the trichloroacetic acid precipitation.

As shown in Table IV, treatment of the cultured cells with cycloheximide for 5 min before labeling the cells with [*Me*-^{3}H]methionine still resulted in significant incorporation of ^{3}H into the peptidyl-tRNA fraction. Samples isolated from the 0.1 *M* NaCl eluate and from the 1.0 *M* eluate following chromatography on the ECTEOLA-cellulose columns were acid hydrolyzed and the methylated basic amino acid fractions were isolated and analyzed for the incorporation of the [^{3}H]methyl group. The peptidyl-tRNA containing fraction prepared from the cycloheximide-treated cells had 22.4% of the level of radioactivity found in the same fraction prepared from the control cells. As seen in Figure 5, the major radioactive amino acids associated with the peptidyl-tRNA fractions in the control (Figure 5A) are Lys(Me), Lys(Me_3), His(3-Me), Arg(G-Me_2), and Arg(G-Me), while the cycloheximide-treated peptidyl-tRNA (Figure 5B) has the highest incorporation in Arg(G-Me_2) and much lower incorporation in Lys(Me_3) and His(3-Me).

Discussion

We have shown that methylation of basic amino acid residues occurs while the protein being methylated is still associated with the ribosomes, a conclusion supported by the results of direct analysis of nascent polypeptides obtained from polysomes of cultured chick skeletal muscle. Further, this methylation does not appear to be dependent on concomitant peptide bond formation, except to provide new peptides as substrates for methylation, since it will proceed on nascent chains in the presence of 0.1 m*M* cycloheximide. This concentration produces a 97.5% inhibition of protein synthesis. In similar experiments performed with rat

muscle cultures, Reporter (1973b) found little or no inhibition of methylation using 0.2 μM cycloheximide, a level producing a 60% inhibition of protein synthesis in his system, and less than 50% inhibition in our experiments.

Methylated amino acid residues have been found in ribosomal proteins (Comb et al., 1966; Terhorst et al., 1972; Reporter, 1973a; Alix and Hayes, 1974; Chang and Chang, 1974; Chang et al., 1974). However, the methylation of ribosome-associated proteins which we observed in the presence of cycloheximide is occurring on nascent peptides or on recently completed proteins still associated with the ribosome and not on proteins of the ribosome itself. This is apparent from the results of the pulse-chase experiment where residues, methylated in the presence of cycloheximide in the pulse part of the experiment, were released from the ribosomes after protein synthesis was allowed to resume with the removal of cycloheximide in the chase.

Cioli and Lennox (1973a) have found terminated proteins, which are defined operationally as polypeptides which are not covalently bound to tRNA, still absorbed on to the ribosomes. Our finding that puromycin releases peptides containing methylated residues argues against the possibility that only the completed protein adsorbed to the ribosomes or alternatively, only ribosomal proteins, are being methylated. A similar conclusion was reached by Reporter (1973b). But polypeptides newly released by puromycin cannot be readily separated from other polypeptides for direct analysis and the puromycin experiments are further complicated by the incorporation of labeled methionine into peptide linkage. However, the decrease in methylated residues in proteins from the polysome region of the sucrose gradients in the puromycin-treated cells suggests that methylated residues were released by puromycin. It should be noted that, while there was a decrease in methylated residues in the polysome region to 54% of the control level, the radioactivity in methylated residues in the non-polysome region of the gradient increased from 45,977 cpm in the control to 84,789 cpm in the presence of puromycin. The methylation of basic amino acid residues could still proceed in the supernatant after puromycin was added, indeed even during ultracentrifugation of the sucrose gradient, since the 12,000g supernatant fraction contains protein methylase activity (I. R. McManus and R. K. Morse, unpublished observations) and labeled S-adenosylmethionine would presumably persist for the duration of the experiment. The release of the nascent chains by puromycin could make more sites on the peptides available for methylation, a reasonable speculation if the proximity of the site of methylation on the peptide to the P site on the ribosome served to limit access of the methylase to its substrate.

It is apparent that the clearest demonstration of methylation of the nascent peptides in the muscle cells comes from direct analysis of the isolated peptides for the presence of methylated residues. The method of Cioli and Lennox (1973a) for the isolation of peptidyl-tRNA has several advantages over previous methods and over the use of the relatively undefined biological approaches. In the method used, the exposure of twice washed ribosomes to sodium dodecyl sulfate and urea dissociates any macromolecules not covalently bound together. Since the nascent peptide is covalently linked to tRNA (Gilbert, 1963; Lapidot and de Groot, 1972), the peptidyl-tRNA can be separated from all other peptides because it is bound to ECTEOLA-cellulose via its tRNA moiety at low ionic strength. Only the peptides constituting the peptidyl-tRNA fraction are known to be covalently attached to polynucleotides. The results of the chromatographic analysis of the isolated peptidyl-tRNA examined both before and after treatment with pancreatic ribonuclease show that methylation of basic amino acids occurs on the nascent tRNA bound peptides and demonstrates the utility of the method for studying the secondary modification of nascent polypeptides.

Similar questions have been asked about secondary modification at the level of nascent chains in the examination of the hydroxylation of prolyl and lysyl residues in collagen. In fact, experiments which have demonstrated that prolyl residues in nascent chains of procollagen are hydroxylated (Manner et al., 1967; Miller and Udenfriend, 1970; Lazarides et al., 1971) have been useful models for our methylation studies. However, their conclusions were controversial (Prockop, 1970; Udenfriend, 1970) until the direct demonstration of the presence of hydroxyproline and hydroxylysine in peptides smaller than the pro-α chains of procollagen (Uitto and Prockop, 1974).

However, there are limits to the analogy between hydroxylation of proline in collagen and methylation of basic amino acids in other proteins. The proline in the sequence Gly-X-Pro in the α-1 subunit of rat skin collagen is hydroxylated in 46 out of the 49 positions in which it appears in a 419-residue fragment (Dayhoff, 1973). It could be said that proline hydroxylase sees its unhydroxylated substrate as a homopolymer because of the large number of repeating sequences which are modified. Thus, it is not unexpected that hydroxylation can be inhibited on nascent peptides of procollagen and these unhydroxylated proteins can then by hydroxylated normally in vivo after release from the polysomes (Lukens, 1970; Lazarides and Lukens, 1971).

In contrast to collagen, methylated residues occur with a much lower frequency in those proteins where they are found (Paik and Kim, 1971). Further, the sequences around His(3-Me) in rabbit skeletal muscle actin and myosin, for example, show no similarity to one another (Elzinga, 1971; Huszar and Elzinga, 1971). The uniqueness and specificity of the sites subject to methylation in a protein suggest that recognition of the site by the appropriate methylase, and access to the site by both the methylase and S-adenosylmethionine, may be subject to parameters less readily defined than simply the primary sequence around the site. Since it has been shown by us and by Reporter (1973b) that nascent chains are methylated, the question is raised as to whether methylation at this stage could be obligatory in the case of some residues in a particular protein. Such a situation could obtain because of steric hindrance to methylation which might occur after release of the completed protein, when it is free to approach its final tertiary structure (Wetlaufer and Ristow, 1973). It is clear, however, that not all of the proteins of the cell are methylated while still being synthesized on the ribosome since our results also show that there is significant methylation of completed proteins in the soluble fraction even in the presence of cycloheximide. It is of interest that the formation of Lys(Me_3) in the cytochrome c of *Neurospora* occurs after termination of synthesis of the protein. The complete, nonmethylated protein is present in the cell and it is the precursor of methylated cytochrome c (Scott and Mitchell, 1969).

At this time, although methylation represents a significant and demonstrable example of the secondary modification of proteins, there is little understanding of its function or of its selective advantage to the organism. Methylation and other post-translational modifications may be manifes-

tations of either a reversible regulatory process or of an irreversible developmental process. Several other authors have speculated on the significance of methylation as a physiological and developmental process (Trayer and Perry, 1966; Holzer and Duntze, 1971; Paik and Kim, 1971; Nickerson, 1973; Segal, 1973). Changes in the degree of methylation have been observed in the contractile proteins of developing muscle (Trayer et al., 1968; Krzysik et al., 1971; Reporter and Corbin, 1971) and in histones of synchronized cultures of mammalian cells (Shepherd et al., 1971; Lee et al., 1973). In terms of developmental processes, some cases of protein methylation may represent a type of "pleiotropic" control over the function of several proteins in the cell at once (see Hersko et al., 1971), or, alternatively, another level of "cascade regulation" of gene expression (see Scherrer and Marcaud, 1968; Scherrer, 1974). It is apparent that the biological significance of protein methylation and particularly of methylation of contractile proteins in muscle is obscure and, although some suggestive evidence exists (Paik and Kim, 1971), an understanding of the role of the methylation of proteins together with full comprehension of the methylation process in the cell remains as a future goal.

Acknowledgments

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Conformational and Functional Studies of Chemically Modified Cytochrome *c*: Nitrated and Iodinated Cytochromes *c*†

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ABSTRACT: The purification of iodinated (E. B. McGowan and E. Stellwagen (1970), *Biochemistry 9,* 3047) and of nitrated (M. Sokolovsky et al. (1970), *Biochemistry 9,* 5113) cytochromes *c* resulted in the recovery from the former preparation of diiodotityrosyl-cytochrome *c* (DIDT-) with modification of Tyr-67 and Tyr-74, and, from the latter, a mononitromonotyrosyl-cytochrome *c* (MNMT-), with modification of Tyr-67, and mononitrodityrosyl-cytochrome *c* (MNDT-), with the added modification of Tyr-48. The three purified preparations were conformationally characterized using pH-spectroscopy, circular dichroism, thermal denaturation, reducibility with ascorbate, autoxidation with molecular oxygen, and binding with CO. These results are related to the two aspects of biological function, reducibility, measured by NADH-cytochrome *c* reductase, and oxidizability, with cytochrome *c* oxidase, as well as to structure-function relationships in the protein. MNMT-cytochrome *c* was found to be, structurally and conformationally, a single isomer, reducible with ascorbate, with a small, but definite affinity for both oxidation with molecular oxygen and binding of CO. Conformationally, in both valence states of the metal atom, it represents a molecular form with native-like conformation with small but definite perturbations in the immediate vicinity of the heme group, reflected by the destabilization of the Met-80-S-Fe linkage. MNMT-ferricytochrome *c* exhibits a pK of 6.2 for the transformation of the low-spin, native-like spectral form II containing the 695-nm band to a form lacking the 695-nm band. The isomerization at pK = 6.2, when analyzed in terms of the isomerization of the native protein with a pK of 9.2 and the nature of the group involved, indicates that Tyr-67 is not involved in the isomerization of the modified preparation, and possibly not in the native protein as well. In terms of biological function, the partial derangement of reducibility (24%) and the unaltered oxidizability point to the functional significance of Tyr-67, and provide another example of selectivity between the two aspects of physiological function, in agreement with the two-function, two-path operational model of the protein. The MNDT- and DIDT-ferricytochromes *c* exhibited physicochemical properties indicative of gross derangement of both the conformation of the protein as well as of the coordination configuration of the metal atom. The complete inability to accept an electron from NADH-cytochrome *c* reductase in both cases, and the retention of 50% of the oxidizability property of DIDT-cytochrome *c*, were interpreted to be the result of conformational derangement, rather than the added modification of Tyr-48 or of Tyr-74.

The functional role of the tyrosyl side chains in horse heart cytochrome *c* has been studied by different laboratories through the modification of functional groups by a variety of group-specific reagents. Acetylation results in modification of two of the four tyrosyl side chains with concurrent alteration of the stimulation of oxygen uptake in depleted mitochondrial preparations (Ulmer, 1966; Cronin and Harbury, 1965). The nitration with tetranitromethane has been shown to result in preferential modification of Tyr-67, although Tyr-48 (Skov et al, 1969) and/or Tyr-74 are also altered (Schejter and Sokolovsky, 1969; Sokolovsky et al., 1970). Iodination, on the other hand, yields a preparation with the modification of Tyr-67 and Tyr-74 (McGowan and

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