Cytochrome *c*-Specific Protein Methylase III from *Neurospora crassa*

By SAMUEL NOCHUMSON, EGON DURBAN, SANGDUK KIM and WOON KI PAIK
*Fels Research Institute and Department of Biochemistry, Temple University School of Medicine,
Philadelphia, PA 19140, U.S.A.*

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A protein methylase III responsible for specifically methylating the cytochrome *c* in *Neurospora crassa* was partially characterized by using unmethylated horse heart cytochrome *c* as a substrate. This enzyme utilizes *S*-adenosyl-L-methionine as the methyl donor. An analysis of the distribution of [¹⁴C]methyl groups in the peptides obtained by chymotrypsin digestion of the enzymically methylated cytochrome *c* showed that all of the radioactivity could be recovered within a single peak after chromatography. This indicates that the enzyme methylates a specific amino acid sequence within cytochrome *c*. On hydrolysis of the radioactive chymotryptic peptide, *Me*-¹⁴C-labelled ϵ -*N*-mono-methyl-lysine, ϵ -*N*-dimethyl-lysine and ϵ -*N*-trimethyl-lysine were identified. The enzyme can easily be extracted from the *N. crassa* mycelial pads and was purified approx. 30-fold.

ϵ -*N*-Methylated lysine residues have been found to occur naturally in such diverse proteins as myosin, actin, flagellin, ribosomal proteins, opsin and cytochrome *c* (Paik & Kim, 1975). Methylation of proteins is a post-translational event and is catalysed by a number of *S*-adenosyl-L-methionine-utilizing enzymes (Paik & Kim, 1971). Although protein methylation is widespread in Nature, its biological significance has not been determined (Paik & Kim, 1971, 1975).

In 1969, the presence of ϵ -*N*-trimethyl-lysine was reported in cytochrome *c* isolated from both wheat-germ and from *Neurospora crassa* (DeLange *et al.*, 1969). Since that time this methylated amino acid has been found to be a constituent of the cytochrome *c* isolated from a number of fungal and higher-plant sources (Boulter *et al.*, 1970; DeLange *et al.*, 1970). The nature of the enzyme system responsible for methylating cytochrome *c* has not yet been described. However, it has been shown that the conversion of unmethylated cytochrome *c* into the methylated form occurs at the protein level in *N. crassa* and that lysine-72 only is modified to ϵ -*N*-trimethyl-lysine (Scott & Mitchell, 1969). Thus *N. crassa* has a potential for being a model system in which to study the relationship between protein methylation and its physiological significance to an organism. In an effort to understand the function of the methylation of cytochrome *c*, a characterization of the *N. crassa* protein(lysine) methyltransferase [*S*-adenosyl-L-methionine – protein(lysine) *N*-methyltransferase; protein methylase III; EC 2.1.1.43] was undertaken.

Experimental

Materials

S-Adenosyl-L-[*Me*-¹⁴C]methionine (sp. radioactivity 55 mCi/mmol) and ACS scintillation solution were purchased from Amersham-Searle, Arlington Heights, IL, U.S.A. Sephadex G-25, horse heart cytochrome *c* (type VI), α -chymotrypsin and the proteins used in the specificity study were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Bio-Rex 70 was a product of Bio-Rad Laboratories, Rockville Centre, New York, N.Y., U.S.A. The *N. crassa* used in these experiments was a peach (*pe*) mutant which was a generous gift of Dr. L. Brodsky, Hahnemann Medical College, Philadelphia, PA, U.S.A. All other reagents were of the highest grade available and obtained from local distributors.

Methods

Growth of *Neurospora crassa*. Stock cultures of *Neurospora crassa* were maintained on slants of *Neurospora* culture agar (Difco, Detroit, MI, U.S.A.). The cultures were grown under sterile conditions in 2.8-litre Fernbach flasks containing 700 ml of Vogel's medium (Davis & DeSerres, 1970). Inoculation was performed directly from the agar slant into the culture flask by using a sterilized platinum loop. The cultures were grown at 30°C with constant shaking for approx. 5 days. The mycelia were harvested by filtration on a Buchner funnel,

followed by washing with 500ml of cold deionized water. Finally the mycelial pad was freeze-dried and stored (desiccated) at -20°C .

Enzyme assay. The assay procedure was a modification of a previously described method (Paik & Kim, 1970).

Duplicate reaction mixtures in 12ml conical test tubes contained $50\mu\text{mol}$ of Tris/HCl, pH 9.0, 1.5mg of horse heart cytochrome *c*, 20nmol of *S*-adenosyl-L-[$\text{Me-}^{14}\text{C}$]methionine (80 c.p.m./pmol), and a portion of the enzyme preparation (100–200 μg of protein) in a final volume of 0.5ml. The blank consisted of the same components, except that the enzyme preparation was first inactivated by being placed in a boiling-water bath for 5 min.

After preincubation of the mixture for 5 min, the reaction was initiated by the addition of the *S*-adenosyl-L-[$\text{Me-}^{14}\text{C}$]methionine and allowed to proceed for 5 min at 37°C . After this time, 0.5ml of 15% (w/v) trichloroacetic acid was added to stop the reaction, and the tubes were covered with marbles and heated in a 90°C water bath for 15 min to precipitate nucleic acids. After cooling, the tubes were centrifuged for 10 min in a clinical centrifuge (IEC) at maximum speed. The supernatant was discarded and the precipitate was washed with two 10ml portions of 7.5% (w/v) trichloroacetic acid by using a pointed glass stirring rod to disperse the pellet. The precipitate was then suspended in 10ml of 95% (v/v) ethanol and heated at 70°C for 10 min. After cooling, the tubes were centrifuged for 10 min in the clinical centrifuge at maximum speed, and the supernatant was discarded. Then 1 ml of 0.2M-NaOH was added to each tube and they were then placed uncovered in a boiling-water bath for 1 h to remove any ω -methyl-arginine and carboxyl methyl esters that may have been formed. [This treatment was used as a routine throughout these experiments, but it has subsequently been found to be unnecessary in this case, since amino acid analysis revealed that ε -*N*-methylated lysine derivatives are the only products formed at each stage of enzyme purification. Also, in assaying for the formation of methanol (Jamaluddin *et al.*, 1976), to determine whether or not carboxy-methyl esters were formed, it was found that under the assay conditions described, no radioactive methanol was produced.] After cooling, 0.4 ml of 0.5M-HCl was added for neutralization, 0.1 ml of 30% (v/v) H_2O_2 was added and then the tubes were heated at 70°C until the reddish-brown colour disappeared (approx. 5 min). This step decreased the amount of colour quenching during liquid-scintillation counting. The contents of the tubes were quantitatively transferred to scintillation vials containing 10ml of scintillation fluid and counted for radioactivity on the ^{14}C channel of a Packard Tri-Carb liquid-scintillation counter with 85–90% efficiency. The specific activity of the enzyme is expressed as pmol of $\text{Me-}^{14}\text{C}$ -

labelled groups incorporated into cytochrome *c*/min per mg of protein.

Subcellular fractionation

A modification of the procedure of Luck (1963) was used for the subcellular fractionation of *N. crassa*. Approx. 10g of *N. crassa* mycelia was collected by filtration on a Buchner funnel and washed several times with a solution of cold 0.44M-sucrose containing 1mM-EDTA (pH 7.4). The washed mycelia were then ground with 20g of dry acid-washed sand in a mortar and pestle and a sufficient amount of the sucrose/EDTA solution was added for a smooth paste to be formed. The paste was filtered through three layers of cheesecloth and washed with 50ml of cold sucrose/EDTA solution. This filtrate constituted the whole homogenate. The whole homogenate was centrifuged at 2000g for 10 min, yielding a crude nuclear pellet and a postnuclear supernatant. The nuclear pellet was washed once with 5ml of sucrose/EDTA, centrifuged at 2000g for 10 min, and the resulting supernatant was added to the first post-nuclear supernatant. The pooled supernatants were then centrifuged at 11000g for 20 min, yielding a crude mitochondrial pellet and a postmitochondrial supernatant. The crude mitochondrial pellet was washed with 5ml of the sucrose/EDTA and again centrifuged at 11000g for 20 min. The supernatant from the mitochondrial wash was combined with the original postmitochondrial supernatant and then centrifuged at 105000g for 1 h. This step resulted in a crude microsomal pellet and cytosolic supernatant having a lipid surface layer. All three crude organelle pellets (nuclear, mitochondrial and microsomal) were resuspended in 1 ml of the sucrose/EDTA solution. The lipid layer from the clear supernatant was removed with a Pasteur pipette and kept. All fractions were then assayed in duplicate for cytochrome *c*-methylating activity as described above. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Partial purification of cytochrome *c* methylase

All procedures were performed at $0-4^{\circ}\text{C}$.

Extraction. *N. crassa*, grown as described above, was harvested by filtering off the medium on a Buchner funnel. The mycelial pad was washed several times with cold water and 3–5g wet wt. was homogenized with 5vol. of deionized water in an electrically driven glass homogenizer (1200 rev./min) until a uniform suspension was obtained. The homogenate was then centrifuged for 1 h at 105000g, the lipid layer was removed from the top by aspiration and the remaining supernatant was kept.

$(\text{NH}_4)_2\text{SO}_4$ precipitation. A saturated solution of $(\text{NH}_4)_2\text{SO}_4$ at room temperature (22°C) was slowly added to the above supernatant with constant stirring until a final concentration of 45% (v/v)

(NH₄)₂SO₄ was obtained. After standing for 30 min, the precipitate was collected by centrifugation at 39 000g for 20 min and the supernatant was discarded.

Calcium phosphate-gel treatment. The (NH₄)₂SO₄ precipitate was dissolved in 5 ml of 0.01 M-sodium phosphate buffer, pH 7.4, and then 5 ml of a suspension of calcium phosphate gel (17 mg/ml of water) was added with careful mixing. After standing for 15 min the gel was removed from the suspension by centrifugation at 3000g for 5 min. The gel was washed once with 40 ml of 0.01 M-sodium phosphate buffer, pH 7.4, and then resuspended in 2.4 ml of 0.03 M-sodium phosphate buffer, pH 7.4. After standing for 10 min, the gel was removed by centrifugation at 39 000g for 5 min and the supernatant containing the enzyme was kept.

Analysis of the enzymically methylated cytochrome c

Preparation of methylated cytochrome c. Horse cytochrome *c* (30 mg) was methylated by using the enzyme fraction eluted from the calcium phosphate gel. The reaction mixture contained 0.5 ml of 0.5 M-Tris/HCl buffer, pH 9.0, 1 ml of cytochrome *c* (30 mg/ml), 1 ml of the enzyme preparation (0.4 mg/ml; activity 280 pmol of [Me-¹⁴C] groups/min per mg of protein) and 0.5 ml of *S*-adenosyl-L-[Me-¹⁴C]methionine (50 nmol/ml; 80 c.p.m./pmol). After incubation for 1 h at 37°C, an additional 0.5 ml of enzyme and 0.5 ml of *S*-adenosyl-L-[Me-¹⁴C]methionine was added and the reaction allowed to proceed for 1 h more. To remove unchanged *S*-adenosyl-L-[Me-¹⁴C]methionine, the reaction mixture was passed through a column of Sephadex G-25 (1.3 cm × 75 cm) equilibrated with deionized water, and the void-volume fractions containing methylated cytochrome *c* were pooled and freeze-dried. The freeze-dried product was dissolved in 2 ml of 50 mM-potassium phosphate buffer, pH 7.0, containing 0.1 mM-K₃Fe(CN)₆ and applied to a column of Bio-Rex 70 (1 cm × 16 cm). Elution was carried out by using a salt gradient formed from 150 ml of 50 mM-potassium phosphate buffer/0.1 mM-K₃Fe(CN)₆, pH 7.0, and 150 ml of 50 mM-potassium phosphate/0.1 mM-K₃Fe(CN)₆/0.5 M-NaCl, pH 7.0. The flow rate was 25 ml/h, and 4 ml fractions were collected. The fractions were monitored by measuring the cytochrome *c* α -band at *A*₅₅₀ and the protein at *A*₂₈₀ as well as determining the radioactivity in each fraction. The fractions containing the radioactive cytochrome *c* were pooled and freeze-dried (see Fig. 2 below). The freeze-dried samples were dissolved in 3 ml of water and desalted by passing through a water-equilibrated column (1 cm × 75 cm) of Sephadex G-25. The radioactive fractions were pooled and freeze-dried.

Digestion of cytochrome c by chymotrypsin. The labelled cytochrome *c* was denatured by the procedure of Margoliash *et al.* (1962). The freeze-dried methylated cytochrome *c* (6.3 mg) was dissolved in

2 ml of deionized water, then 8 ml of ethanol was added, the sample was mixed and left for 24 h. After this time the denatured methylated cytochrome *c* was removed from suspension by centrifugation in the clinical centrifuge at maximum speed and then washed with three 10 ml portions of ethanol. The sample was air-dried and subjected to enzymic digestion by α -chymotrypsin by the method of Margoliash & Smith (1962). After a 30 h hydrolysis, which included three additions of α -chymotrypsin (1:50 molar ratio to cytochrome *c*) at 0, 9 and 20 h, the reaction mixture was freeze-dried and resuspended in 2 ml of deionized water, and some remaining insoluble material was removed by centrifugation. The radioactivity in the sample was then determined.

Analysis of the chymotryptic peptides. The chymotryptic peptides were analysed on a column of Dowex 50-X2 by a modification of the procedure described by Margoliash & Smith (1962). A portion of the chymotryptic digest (33 000 d.p.m.) was applied to a column (0.9 cm × 64 cm) of Dowex 50-X2 (200–400 mesh) and eluted with approx. 800 ml of 0.2 M-pyridine/acetate buffer (pH 3.0) at 40°C with a flow rate of 30 ml/h maintained by a constant-volume-delivery pump (Mini-pump; Milton Roy Co., Philadelphia, PA, U.S.A.). After completion of the elution by this buffer, a pH gradient was formed from 500 ml of 0.2 M-pyridine/acetate buffer (pH 3.0) and 500 ml of 2.0 M-pyridine/acetate buffer (pH 5.0). Throughout the analysis the eluate was constantly monitored by using a flow cell packed with anthracene crystals (primary fluor), inserted into the counting chamber of a Packard Tri-Carb liquid-scintillation spectrometer. After the eluate passed through the flow cell it was fractionated into fractions (4.5 ml) by using a Gilson fraction collector. A 0.2 ml portion of every third fraction was analysed after alkaline hydrolysis by using the ninhydrin method (Hirs *et al.*, 1956).

Analysis of the radioactive peptide peak. The fractions under the radioactive peak were pooled and then dried in a rotary flash evaporator (Buchler). The dried sample was dissolved in 2 ml of 6 M-HCl and hydrolysed *in vacuo* for 24 h at 110°C. The hydrolysate was dried in a flash evaporator and redissolved in 0.5 ml of water. The hydrolysate was then analysed for methylated amino acids as described previously (Paik & Kim, 1975).

Results

Determination of some of the kinetic parameters of a crude protein(lysine) methyltransferase

A protein(lysine) methyltransferase having a pH optimum of 9 could easily be extracted from the mycelial pad of *N. crassa* by homogenization in ice-cold deionized water. In addition, most of the enzyme

activity remained in the supernatant after centrifugation of the homogenate at 105000g for 1 h. As shown in Fig. 1(a), the enzyme reaction was linear with respect to time for at least 10 min. Also the reaction velocity was proportional to the soluble protein concentration up to 200 μ g (Fig. 1b).

The soluble enzyme exhibited classical Michaelis-Menten kinetics when the concentration of *S*-adenosyl-L-methionine was varied. An apparent K_m of $9.5(\pm 1.4) \times 10^{-6}$ M was calculated for *S*-adenosyl-L-methionine, which is in the same range of values found for other protein methyltransferases (Paik & Kim, 1971). The dependence of the enzyme reaction on cytochrome *c* concentration was also investigated, and an apparent K_m of $1.20(\pm 0.20) \times 10^{-4}$ M was calculated (Lineweaver & Burk, 1934).

Effect of free lysine on the cytochrome c-methylation reaction

An enzyme which utilizes *S*-adenosyl-L-methionine as a methyl donor to methylate lysine at the free-amino acid level has been described in certain strains of *N. crassa* (Borum *et al.*, 1975). Although we are measuring only the incorporation of a radioactive methyl group into the trichloroacetic acid-precipitable cytochrome *c*, it may be possible that free lysine is actually methylated and in some way is becoming bound to cytochrome *c* and this is the reaction being measured. Therefore the effect of adding increasing concentrations of lysine was determined in an attempt to dilute the amount of radioactivity incorporated into cytochrome *c*. The addition of free lysine up to 10 mM did not dilute the radioactivity incorporated into precipitated cytochrome *c*. This indicates that lysine methylation was not operating under the present reaction conditions and that methylation is occurring at the protein level. In addition, the presence of cycloheximide in the reaction mixtures containing the soluble enzyme had no effect, thereby excluding the possibility of an endogenous amino acid becoming methylated and then being incorporated into a peptide chain.

Subcellular localization of the methylating enzyme

Table 1 shows the result of a subcellular fractionation used in the localization of the enzyme. Since the enzyme was originally easily extractable with water, it was decided to determine whether the activity originated from a cellular organelle or was present in the cytosol. It was found that none of the isolated crude organelle fractions had significant enzyme activity. A surprising finding was that approx. 77% of the protein was found in the cytosolic fraction, along with 88% of the enzyme activity. A small amount of enzyme activity was found in the ribosomal fraction

Fig. 1. (a) Time course for protein(lysine) methyltransferase reaction, and (b) effect of protein concentration on initial velocity

Neurospora crassa cytosol was assayed for cytochrome *c* protein methylase activity as described under 'Methods'.

and may be due to a ribosomal methylating enzyme which has been described in *Escherichia coli* (Chang *et al.*, 1975).

Partial purification of the enzyme

A small-scale purification procedure for the protein(lysine)methyltransferase has been developed

Table 1. *Subcellular distribution of protein(lysine) methyltransferase*

A subcellular fractionation from 10g wet wt. of *Neurospora crassa* mycelia was performed as described under 'Methods'.

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (pmol of [¹⁴ C]methyl groups/min per mg of protein)	Total activity (pmol of [¹⁴ C]methyl groups/min)	Recovery (%)
1. Whole homogenate	18.5	3.3	8.8	538	—
2. Nuclei	1.0	1.0	0	0	0
3. Mitochondria	1.0	1.8	0	0	0
4. Microsomal	1.7	3.7	0.4	2.5	0.5
5. (a) lipid layer	4.8	1.7	9.0	73	13.6
(b) cytosol	16.5	2.4	10.1	400	74.3

Table 2. *Partial purification of Neurospora crassa protein(lysine) methyltransferase*

A 5g portion of *N. crassa* mycelia was homogenized in 5vol. of water to extract the cytochrome *c*-specific protein methylase III and the homogenate was fractionated as described under 'Methods'.

Enzyme fraction	Volume (ml)	Protein (mg/ml)	Specific activity (pmol of [¹⁴ C]methyl groups/min per mg of protein)	Yield (%)	Purification (fold)
1. Whole homogenate	16.0	6.1	9.0	100	—
2. 105000g supernatant	12.8	4.1	13.4	80	1.5
3. 0–45% (v/v) (NH ₄) ₂ SO ₄	5.0	2.4	36.1	49.3	4.0
4. Eluate from calcium phosphate gel	1.0	0.29	289	9.6	32.1

Table 3. *Substrate specificity*

A portion (2mg) of each of the listed substrates was incubated with *Neurospora crassa* cytosol (200 µg) and assayed for their methyl-accepting activity as described under 'Methods'. Values are expressed as a percentage of the activity obtained with cytochrome *c*. Bovine serum albumin, γ-globulin, fibrinogen and insulin had no methyl-accepting activity.

Substrate	Relative activity
Horse heart cytochrome <i>c</i>	100
Myoglobin	10.4
Haemoglobin	7.4
Histone:	
Sigma II-A	2.9
F1	6.5
F2a	0.0
F2b	0.4
F3	0.0
Ribonuclease	4.7
Egg-white globin	1.7
Trypsin inhibitor	1.0
Polylysine	0.2

The enzyme preparation after (NH₄)₂SO₄ precipitation and elution from the calcium phosphate gel contains no free amino acids, and this is further evidence that methylation is occurring at the protein level.

Specificity of the protein(lysine) methyltransferase

Table 3 indicates that the protein-methylating activity found in the 105000g supernatant of water-extracted *N. crassa* is relatively specific for horse heart cytochrome *c*. Thus the enzyme when purified may have an absolute specificity toward cytochrome *c*. This is in contrast with the rat liver nuclear protein methylase III, which has a broad specificity for mainly histones and no activity towards cytochrome *c* (Paik & Kim, 1970).

Since horse heart cytochrome *c* has 19 lysine residues, it was decided to investigate the distribution of the incorporated radioactive methyl group to find out if it was random or selective. Thus the methylated product was further characterized after digestion by α-chymotrypsin in an attempt to identify which chymotryptic peptide(s) of the horse heart cytochrome *c* was accepting the radioactive methyl group, and to determine whether or not lysine is the only amino acid being methylated. An elution profile of the methylated cytochrome *c* on a column of Bio-Rex 70 (Fig. 2) shows that methylation results in a species which is bound less tightly to the column than the unchanged cytochrome *c*. Further, after chymo-

to give a 32-fold increase in specific activity with a 10% yield (Table 2). The enzyme may be extracted from either early or old cultures of *N. crassa* without any significant difference in the specific activity. For convenience the mycelial pad may be freeze-dried and stored desiccated at –20°C until needed.

Fig. 2. Elution profile of enzymically methylated cytochrome *c*

After reaction with the *Neurospora crassa* protein-(lysine)methyltransferase, 30 mg of cytochrome *c* was applied to a column (1 cm × 16 cm) of Bio-Rex 70 and eluted with a salt gradient. See under 'Methods' for details. ○, Cytochrome *c* A_{550} ; ●, radioactivity.

trypsin digestion of the methylated cytochrome *c* fraction, an analysis of the peptides on a column of Dowex 50 (Fig. 3) shows that all of the radioactivity applied (33000 d.p.m.) was recovered in one peak. This indicates that the enzyme does not randomly methylate cytochrome *c*, but rather exhibits an absolute specificity for some amino acid sequence within the protein. An amino acid analysis of the pooled radioactive fractions after acid hydrolysis revealed only the presence of three methylated lysine derivatives (Fig. 4). The distribution of radioactivity

Fig. 4. Amino acid analysis of the isolated radioactive peptide fraction obtained from α -chymotrypsin-treated methylated cytochrome *c*

The radioactive peak in Fig. 3 was collected and the material hydrolysed in 6M-HCl for 24 h at 110°C. The hydrolysate was then analysed on a Perkin-Elmer amino acid analyser as described previously (Paik & Kim, 1975). Radioactive lysine was added as a standard. Abbreviations: Lys, lysine; Me-Lys, ϵ -N-monomethyl-lysine; Me₂-Lys, ϵ -N-dimethyl-lysine; Me₃-Lys, ϵ -N-trimethyl-lysine.

Fig. 3. Separation of chymotryptic peptides of enzymically methylated cytochrome *c*

Methylated cytochrome *c* isolated as a peak from the column of Bio-Rex 70 was treated with α -chymotrypsin, and ^{14}C radioactivity (33000 d.p.m.) was applied to a column (0.9 cm × 64 cm) of Dowex 50-X2 and eluted with pyridine/acetate buffer as described under 'Methods'. The flow rate was 30 ml/h and fractions (4.5 ml) were collected. ●, Ninhydrin A_{570} ; △, radioactivity; ○, pH.

was as follows: 9% in ϵ -*N*-monomethyl-lysine, 36% in ϵ -*N*-dimethyl-lysine and 55% in ϵ -trimethyl-lysine.

Discussion

A protein(lysine)methylating enzyme which is highly specific for cytochrome *c* has been found in the fungus *Neurospora crassa*. This relatively high specificity is unlike other protein methylases, which usually exhibit methylating activity towards a wide variety of proteins (Kim & Paik, 1970; Kim, 1974; Greenaway & Levine, 1974); therefore the *N. crassa* enzyme would probably remain undetected unless unmethylated cytochrome *c* was used as the substrate. This example emphasizes the importance of having the proper protein substrate for enzyme identification and probably indicates that a number of specific protein methylases have yet to be described.

The *N. crassa* enzyme has been localized within the cytosol. It can easily be extracted from the mycelial pad by homogenizing in water, in marked contrast with the rat liver chromatin enzyme, which is freed by treatment with detergent (Paik & Kim, 1970). However, it is similar to the *E. coli* ribosomal protein methylase, which is readily soluble (Chang *et al.*, 1975). The localization of the enzyme in the cytosol suggests that methylation occurs before translocation of the cytochrome *c* into the mitochondria.

The *N. crassa* cytochrome *c* has been shown to contain one trimethyl-lysine residue at position 72 (DeLange *et al.*, 1969) out of 14 possible sites for lysine methylation (Heller & Smith, 1966). This specificity may be due to conformational restrictions imposed on the enzyme and/or cytochrome *c* by some process *in vivo*, thereby influencing the specific lysine residue which is available for methylation. Since we are examining a situation *in vitro* by using horse cytochrome *c* as the methyl acceptor, we decided to determine whether the pattern of methylation of this haemoprotein was random or if the enzyme maintained a similar selectivity to that found for the *N. crassa* cytochrome *c*. When the enzymically methylated cytochrome *c* was chromatographed on a basic-ion-exchange resin (Fig. 2), the radioactive modified cytochrome *c* was eluted before the unmodified substrate. This result is analogous to the observation by Scott & Mitchell (1969) in which the methylated cytochrome *c* of *N. crassa* was less tightly bound to the basic ion-exchange resin than the unmethylated cytochrome *c*.

After chymotryptic digestion of the enzymically labelled methylated cytochrome *c*, a peptide-elution pattern from a Dowex 50 column (Fig. 3) resulted in one radioactive peak in which all of the applied radioactivity was recovered. The location of this peak appears to be in a similar position as judged by pH

to that of peptide XXIV, which corresponds to residues 68–74 in horse heart cytochrome *c* (Margoliash & Smith, 1962). Since the peptide contains two lysine residues, at positions 72 and 73, the exact lysine position that is methylated requires further verification.

Unfortunately the amount of material recovered was insufficient for further peptide purification and determination of the amino acid composition. However, the fact that all of the radioactivity was eluted in a single peak indicates that the enzyme is specific for a particular lysine-containing sequence in the horse heart cytochrome *c*. It is noteworthy that the lysine residues in horse heart cytochrome *c* are all located on the outside of the molecule (Dickerson *et al.*, 1971) and are therefore candidates for enzymic methylation. The fact that one particular lysine-containing sequence is methylated suggests that the recognition site for the protein(lysine)methyltransferase may be determined by both amino acid sequence and conformation.

After acid hydrolysis of the isolated radioactive-peptide peak (Fig. 3), amino acid analysis revealed that ϵ -*N*-monomethyl-lysine, ϵ -*N*-dimethyl-lysine and ϵ -*N*-trimethyl-lysine were present in the molar proportions 1:4:6 (Fig. 4). The presence of ϵ -*N*-monomethyl-lysine and ϵ -*N*-dimethyl-lysine probably reflects intermediate products in the formation of ϵ -*N*-trimethyl-lysine, since the reaction did not go to completion, as indicated by a substantial amount of unmethylated cytochrome *c* that was eluted from the ion-exchange column (Fig. 2). Another possible explanation for the presence of all three methylated lysine products is that there is more than one methylating enzyme, each responsible for the addition of a methyl group to the lysine residue.

An investigation of cytochrome *c* methylation is of interest from an evolutionary perspective. The amino acid sequence from residues 70 to 80 in cytochrome *c* is strictly conserved throughout evolution (Margoliash & Schejter, 1966) except for lysine-72, which has been modified in lower organisms to trimethyl-lysine. Why a methylated lysine residue at position 72 in the cytochrome *c* of certain organisms is of such importance that they require an additional energy-requiring protein-methylation reaction, including the synthesis of a specific methylating enzyme, requires investigation, as does the reason for the deletion of this protein-modification reaction in higher organisms. Further, plants have another trimethylated lysine residue at position 86 (Boulter *et al.*, 1970; DeLange *et al.*, 1970). This finding implies that either there are two enzymes, each specific for one of the lysine residues, or there is one enzyme responsible for methylating both lysine residues.

The fact that cytochrome *c* methylation is not conserved throughout evolution does not mean that protein methylation in general is being deleted, since

it is common in a number of higher species. Thus histone IV is not methylated in plants, but is found methylated in mammals (DeLange & Smith, 1975).

Thus far the investigation of cytochrome *c* methylation in *N. crassa* has been found to offer considerable advantages over mammalian protein-methylation systems. These include: (1) the availability of a well-characterized protein substrate in an unmethylated form, (2) the presence of a soluble protein methylase which is extremely specific for cytochrome *c*, and (3) the possibility of obtaining mutant strains deficient in some aspect of cytochrome *c* methylation to aid in the identification of a function for this protein-modification reaction.

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