must be kept in mind that the titration curve of tetramer would be expected to shift relative to that of the dimer due to a reduction in the electrostatic interaction between protons and the macromolecular species with increasing molecular weight. Thus, the difference in protons bound to the two species is not only a function of metal ion binding but also of the altered titration curve of the protein itself.

The number of binding sites on alkaline phosphatase for the competitive inhibitor, P_i, is 1/dimer under a variety of experimental conditions but increases to 3.6/dimer when 33 mole % of the protein is present as tetramer. Assuming there are only two species present, 0.67 (±0.07) mole of dimer and 0.33 (±0.03) mole of tetramer and, further, that each dimer contains only one site, the simple calculation belows shows that 17 ± 1 moles of P_i are bound to the tetrameric form of the protein. It appears that approximately

$$0.33\left(\frac{x}{2}\right) + 0.67(1) = 3.6$$

$$x = 17.8$$

1 mole of P_i is bound for each mole of Zn^{2+} on the protein in the tetrameric state.

Stopped-flow kinetic experiments comparing the tetramer and dimer as to number of catalytic sites and velocity of reaction will be reported in a subsequent communication.

Although alkaline phosphatase is isolated from *E. coli* cells as a highly stable dimer, it is possible that the ionic conditions in the periplasmic space where the enzyme is localized favor the tetramer form. Under derepressed conditions of growth, there are approximately 10^4 enzyme molecules/cell confined to an estimated space of 10^{-18} cm^3, or about 10 g/l. Experiments are in progress to determine the state of alkaline phosphatase *in situ*.

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Secondary Modification of Cytochrome *c* by *Neurospora crassa**

W. A. Scott† and H. K. Mitchell

ABSTRACT: Two molecular species of cytochrome *c*, C_I and C_{II}, were detected in *Neurospora crassa*. The two cytochrome *c*'s differ in structure at residue 72. This position is occupied by lysine in C_{II}. The equivalent residue in C_I, a lysine derivative, has been identified as ε-trimethyllysine (DeLange, J. R., Glazer, A. N., and Smith, E. L. (1969), *J. Biol. Chem. 244*, 1385). A sequential synthesis of the two cytochrome *c*'s occurs in *Neurospora* cultures. Pulse-chase experiments with [^{14}C]lysine indicate that C_{II}, the early cytochrome *c*, is converted into C_I presumably by a specific methylation of lysine-72 in C_{II} to give ε-trimethyllysine in C_I. The kinetics of C_I and C_{II} synthesis in the *poky* mutant also suggest that the conversion of C_{II} into C_I reflects the binding of the cytochrome *c* to the mitochondrion.

Cytochrome *c* does not normally occur free in the cytoplasm of a cell. Apparently there is coordination between the rate of cytochrome *c* synthesis and its binding to the mitochondrial matrix. However, an exceptional situation exists in the *poky* strain of *Neurospora crassa*. This mutant, which exhibits cytoplasmic inheritance (Mitchell and Mitchell, 1952), accumulates up to 16 times the normal amount of cytochrome *c* found in the *wild-type* strain (Haskins *et al.*, 1953), over half of which is not bound (Hardesty, 1961). This extreme condition exists only in very young cultures. The cytochrome *c* accumulation is diluted out as the mold grows and ages. Using very young cultures and alkaline extraction, Hardesty (1961) obtained evidence that the *poky* mutant contains more than one molecular species of cytochrome *c*.

With this background, the *poky* mutant clearly presented a favorable situation in which to examine the biosynthesis of cytochrome *c* and to investigate further the question of the existence of more than one species of molecule. As reported here, there are indeed two cytochrome *c*'s produced in the mold, one being derived from the other.

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Experimental Procedures

Growth of Neurospora. Conidia were grown under constant illumination in 1-l. wide-mouth erlenmeyer flasks containing 200 ml of complete medium (Horowitz, 1947) plus 1.5% agar. The sides of the flasks were coated with a thin layer of medium by swirling the flasks prior to solidification of the agar. Liquid cultures were grown at 25° in 5-gal Pyrex carboys containing 16 l. of medium N (Vogel, 1956) plus 2% sucrose. All cultures were inoculated with 10^7 conidia/l. of medium. Sterile air was supplied at the rate of 12 l./min. Under these conditions *poky* cultures remained in logarithmic growth for 30 hr. Immediately after terminating aeration, the mold was collected by filtering the medium through three to four layers of cheesecloth. The mycelium was used immediately or frozen at −27°.

Cytochrome c Purification. All steps were performed at 5° except for the Amberlite CG-50 columns which were run at room temperature.

STEP 1. Pressed dry *Neurospora* (200 g) was frozen in liquid nitrogen and ground in a metal Waring blender until a fine powder resulted. After carefully pouring liquid nitrogen over the mycelial powder, it was ground a second time and allowed to thaw at 5°.

STEP 2. The mycelial powder was mixed with 2 ml of pH 8.6 Tris-HCl buffer (0.05 M) per g of tissue and the pH was brought to 10.5 with concentrated ammonium hydroxide. After sitting for 15 min the stirring was started again as the pH was reduced to 8.0 by the dropwise addition of glacial acetic acid. The precipitate, collected by centrifugation at 3000*g* for 10 min, was washed three times with the pH 8.6 buffer. The washings were combined with the original supernatant.

STEP 3. The red supernatant was centrifuged at 79,000*g* for 60 min. The red, opaque precipitate yielded little cytochrome *c* on extraction with ammonia. It was routinely discarded. At this stage the preparation was a clear, red solution. Dialysis was carried out against 100 volumes of 0.005 M phosphate buffer (pH 7.0) containing 10^{-4} M $K_3Fe(CN)_6$.

STEP 4. The cytochrome was adsorbed onto 500 ml of acid-washed Hyflo-Super gel packed on a coarse fritted glass funnel 10 cm in diameter. Suction was employed to maintain a constant flow rate. The adsorbant was washed with several volumes of distilled water and packed into a column. The cytochrome was eluted with 0.5 M NaCl in 0.05 M phosphate buffer (pH 7.0). Dialysis of the cytochrome *c* containing fractions was carried out as in step 3.

STEP 5. A 1.5 × 90 cm column of Amberlite CG-50, treated with hypochlorite (Hagihara *et al.*, 1958), was equilibrated with 0.05 M phosphate buffer (pH 7.0). The cytochrome *c* (50–150 mg) adsorbed as a narrow red band at the top of the resin. Chromatography was carried out at a flow rate of 5.5 ml/hr using a linear gradient with respect to ionic strength, performed between 150 ml of 0.05 M phosphate buffer (pH 7.0) and 150 ml of the same buffer containing 0.5 M NaCl. Both buffers contained 10^{-4} M $K_3Fe(CN)_6$. Fractions of 2 ml were collected. Cytochrome *c* containing fractions were pooled and dialyzed as in step 3.

STEP 6. Cytochrome c was rechromatographed on Amberlite CG-50 under the conditions described in step 5. Fractions containing C_I and C_{II} were pooled separately and dialyzed as in step 3.

STEP 7. Dialyzed samples of C_I and C_{II} were rechromatographed separately on 1.5 × 50 cm columns of Amberlite CG-50 as in step 5. Contents of the appropriate fractions were pooled, dialyzed against the appropriate buffer or distilled water, and frozen at −20°.

Cytochrome c Determination. Cytochrome *c* solutions were routinely scanned in the 590–500-mμ region with a Cary Model 15 recording spectrophotometer after reduction with sodium dithionite. Cytochrome *c* concentrations were estimated from the absorption maximum at 550 mμ assuming an extinction coefficient of 29,000 (Massey, 1959). Ultraviolet spectra were obtained prior to reduction.

Cytochrome *c* in cell extracts and mitochondria was determined by difference spectra (Williams, 1964).

Sedimentation Constants of C_I and C_{II}. Sedimentation constants of C_I and C_{II} were estimated by the band centrifugation technique (Vinograd *et al.*, 1963) in a Model E ultracentrifuge equipped with ultraviolet absorption optics. A standard cell with sapphire windows and a 12-mm Kel-F band-type centerpiece was employed. The lamella consisted of 25 μl of cytochrome *c* (10 mg/ml) in 0.05 M Tris-HCl (pH 7.6) and the bulk solvent was 1 M NaCl in the same buffer. A record of protein concentration *vs.* radius was obtained from the developed film with a Joyce-Loebl double-beam microdensitometer.

Digestion of Cytochrome c with Chymotrypsin. Cytochrome *c* (22 mg of C_{II} and 20 mg of C_I) was denatured with 70% ethanol as described by Matsubara and Smith (1963). The denatured cytochrome *c* was precipitated by centrifugation at top speed in a clinical centrifuge, then redissolved in 5.0 ml of distilled water, and adjusted to pH 8.0 with 0.005 N NaOH. The digestion was performed at room temperature with 2% chymotrypsin (Worthington, twice crystallized). The digestion mixtures were maintained at pH 8.0 with 0.0509 N NaOH by automatic titration in a Radiometer TTTI pH-Stat. After 12 hr an additional 2% chymotrypsin was added. The reaction was terminated after 24 hr by freeze drying.

Separation of Chymotryptic Peptides. The chymotryptic digestion mixture was chromatographed on a 1 × 34 cm column of Dowex 50-X2 at 40° at a flow rate of 17 ml/hr maintained by a constant delivery pump. Fractions of 2 ml were collected. The buffer systems were those described by Matsubara and Smith (1963). From every third fraction 0.2 ml was removed for ninhydrin analysis after alkaline hydrolysis (Hirs *et al.*, 1956). Contents of the tubes from the ninhydrin-positive peaks were pooled, freeze dried, and stored at −20° in 1.0 ml of distilled water.

The number of peptides in each pooled fraction was ascertained by electrophoresis on Whatman No. 3MM paper at pH 3.5 (4.5 hr at 75 V/cm) and at pH 6.0 (8 hr at 26.7 V/cm). The compositions of these buffers were: pH 3.5, 1% pyridine–10% acetic acid; pH 6.0, 20% pyridine–2.5% acetic acid. A combination of chromatography–electrophoresis was also employed. Chromatography (butanol–acetic acid–water, 4:1:5, for 16 hr) was followed by pH 3.5 electrophoresis. Peptides were visualized with the collidine–ninhydrin reagent (Dreyer and Bynum, 1967).

After these tests one-half of each peptide fraction was freeze dried, then dissolved in 30 μl of distilled water, and applied to Whatman No. 3MM paper. By the appropriate electrophoretic conditions the peptides of each pool were separated. Peptides were located by the ninhydrin detection spray

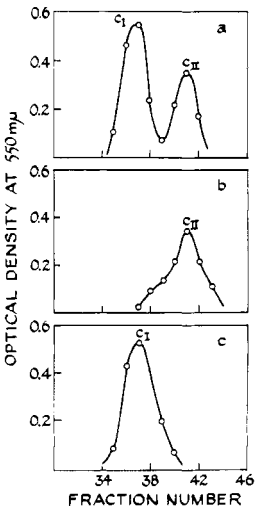

FIGURE 1: Chromatographic separation of C_I and C_{II} on Amberlite CG-50. Cytochrome *c* was extracted from a 40-hr sample of *poky* and purified. (a) Primary chromatography of *poky* cytochrome *c* on a 1 × 10 cm Amberlite CG-50 column. (b) Rechromatography of C_{II} obtained by pooling the appropriate fractions (39–43) from the column in part a. (c) Rechromatography of C_I obtained by pooling fractions 34–38 from the column in part a.

(Dreyer and Bynum, 1967) and eluted from the paper either with constant-boiling HCl or distilled water.

The nomenclature for peptides is similar to that of Heller and Smith (1966). Each chymotryptic peptide is designated by C. Each peptide is given a number according to its position from the N-terminal end of the polypeptide chain (C-1). In cases where distinction is made between equivalent peptides of C_I and C_{II}, the C_I peptide is designated as C_I-1 and the C_{II} peptide as C_{II}-1.

Amino Acid Analysis. Peptides were hydrolyzed in constant-boiling HCl for 24–48 hr at 105° under nitrogen in sealed thick-wall Pyrex tubes. Each hydrolyzed peptide was dried *in vacuo* over KOH pellets and dissolved in 50 μl of distilled water. Usually 20 μl was sufficient for analysis. High-voltage paper electrophoresis was employed for amino acid analysis of peptides as described by Dreyer and Bynum (1967). Standard amino acid mixtures were run alongside of each sample for purposes of calibration. Quantitative estimation of each amino acid was carried out with the cadmium–ninhydrin reagent (Dreyer and Bynum, 1967). Each ninhydrin-positive spot was shredded and the color was eluted in stoppered test tubes with 3.0 ml of absolute methanol by shaking for 1.5 hr at 25°. Amino acid concentrations were calculated from standard curves of optical densities of the methanol supernatants read at 500 mμ.

Column amino acid analysis was a micro version of the procedure of Piez and Morris (1960).

Pulse-Labeling of Neurospora. Freshly collected *Neurospora* was gently pressed between paper towels to remove excess liquid, and then resuspended with gentle agitation in 20 volumes by weight of sterile Vogel's medium N containing 2% sucrose. After adding the radioactive amino acid, the incubation mixture was shaken on a reciprocal shaker. The incorporation was stopped by collecting the mold on a Büchner funnel with suction. The radioactive medium was removed by washing with distilled water. The tissue was ground immediately or frozen at −20°.

Radioactivity Determinations. Radioactivity was determined by liquid scintillation counting with a Nuclear-Chicago 720 Series scintillation counter. Aliquots (0.2 ml) from fractions of Amberlite CG-50 columns containing cytochrome *c* were counted in Bray's solution (Bray, 1960) by the channels ratio method. Quenching due to cytochrome *c* color was estimated by adding varying amounts of cytochrome *c* to known amounts of radioactive amino acids. All reported values are corrected to 60% counting efficiency for ^{14}C and 20% counting efficiency for ^{3}H.

For the determination of ^{3}H in amino acids separated electrophoretically on paper and stained with the cadmium–ninhydrin reagent, the following procedure was used. The methanol extracts were evaporated in scintillation vials with a gentle stream of air after bleaching the red color with two drops of concentrated HCl. Hyamine hydroxide (0.15 ml) was added to neutralize any remaining HCl and to dissolve the residue. The vials were filled with Bray's solution and counted by the channels ratio method. The paper was also counted after the methanol extraction.

Results

Cytochrome *c* was purified 250-fold from crude extracts of *poky* with a 40% yield. The purified protein had a 550 mμ (reduced):280 mμ (oxidized) ratio of 1.2 which is in good agreement with previous results for purified *Neurospora* cytochrome *c* (Heller and Smith, 1966). It was necessary to maintain a basic pH in the initial stages of purification because cytochrome *c* is rapidly destroyed in crude extracts of *poky* under neutral or acidic conditions (Hardesty, 1961). The cytochrome *c* destroying activity was removed by the ammonium sulfate step after which the pH could be lowered to facilitate adherence of the cytochrome *c* to the Amberlite CG-50 resin.

Two Cytochrome c's. Purified cytochrome *c* extracted from exponentially growing cultures of *poky* separates into two peaks on Amberlite CG-50 chromatography as shown in Figure 1a. The two cytochrome *c*'s, designated as C_I and C_{II} according to their order of elution, elute at 0.275 and 0.290 M sodium ion, respectively, which is the same cation concentration range for the elution of native horse heart cytochrome *c* from Amberlite IRC-50 (Margoliash and Schejter, 1966). The separation of C_I and C_{II} is highly dependent upon the flow rate of the column. At a flow rate of 5.5 ml/hr, C_I and C_{II} separate into distinct peaks; however, at higher flow rates a broad cytochrome *c* peak is obtained with only partial separation of the two proteins.

Chromatography of C_I and C_{II} separately on Amberlite CG-50 gave the elution profiles shown in Figure 1b,c. Each protein elute as a single homogeneous protein at the same cation concentration as in the primary chromatography. The shoulder on the leading edge of the C_{II} peak in Figure 1b is contaminating C_I as shown by cochromatography of the cytochrome *c* from the shoulder with purified C_I. To determine whether the two cytochrome *c*'s are interchangeable on chromatographic columns, C_I and C_{II} were mixed in varying ratios and chromatographed on Amberlite CG-50. The ratio of the eluted C_I and C_{II} was always identical with that of the input mixture.

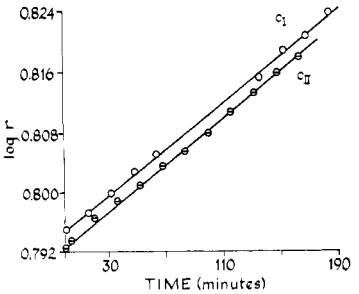

FIGURE 2: Band centrifugation of C_I and C_{II}. Positions of band maxima were measured from densitometer tracings of photographs taken at the indicated times. The logarithms of the distances (centimeters) of the band maxima from the center of rotation are plotted as a function of time.

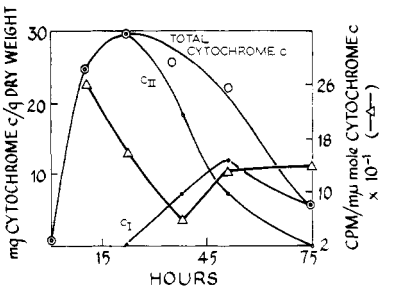

FIGURE 3: Amounts of total cytochrome *c*, C_I, C_{II}, and the specific activities of cytochrome *c* at different ages of *poky*. Each sample of *poky* was incubated for 30 min with 1 μCi of [^{14}C]lysine/g of tissue. Cytochrome *c* is represented by circles. Specific activities are represented by triangles. See the text for details.

Sedimentation Coefficients and Spectra. Sedimentation coefficients of C_I and C_{II} were determined by the band centrifugation technique of Vinograd *et al.* (1963). From a plot of ln *r vs. t* (Figure 2), the sedimentation coefficients were calculated from the formula $\ln r = s\omega^2 t + \text{constant}$. For correction to standard conditions of 20° and water it was assumed that $V_{20} = V_t = 0.721$ (Heller and Smith, 1966) and that $\rho_{t,\text{solvent}} = 1.0385$ and $\rho_{w,20} = 0.99823$ (Svedberg and Petersen, 1940) for both proteins. Both C_I and C_{II} have similar sedimentation coefficients: $s_{20,w}$ C_I = 1.62 S and $s_{20,w}$ C_{II} = 1.66 S.

The ultraviolet and visible absorption spectra of C_I and C_{II} are identical and are similar to those previously published by Heller and Smith (1966). Both proteins are stable to air oxidation when in the reduced state.

Multiple chromatographic forms of cytochrome *c* are found in preparations from almost all sources. Most are attributed to artifacts such as polymeric forms, aggregates with basic proteins, and deamidated monomers that result from harsh alkaline or acidic extraction conditions (Margoliash and Schejter, 1966). The oxidized and reduced forms of cytochrome *c* also separate on ion-exchange columns. The sedimentation and spectral data eliminate the possibility of polymerization or aggregation with basic proteins as the difference between C_I and C_{II}. In addition, a comparison of the electrophoretic mobilities of the comparable C_I and C_{II} chymotryptic peptides at three different pH values (see structural difference between C_I and C_{II}) revealed no significant mobility differences, which would argue against deamidation as a difference. To eliminate separations due to a difference of oxidation state, $K_3Fe(CN)_6$ was included in the gradient buffers. The $K_3Fe(CN)_6$ did not affect the elution characteristics of C_I and C_{II}, but did reduce the amount of reduced cytochrome *c* that routinely occurs during chromatography. (Reduced cytochrome *c* elutes as a distinct peak from Amberlite CG-50 prior to oxidized C_I and C_{II}.)

C_I and C_{II} Synthesis. The rate of cytochrome *c* synthesis, the total amount of cytochrome *c* and the amounts of C_I and C_{II} were determined at different ages of *poky*. Cytochrome *c* was purified from samples of *poky* previously incubated for 30 min with 1 μCi of [U-^{14}C]lysine/g wet weight of cells (see Experimental Procedures). Total cytochrome *c* was estimated in the supernatant and precipitate of step 2 of the purification procedure by difference spectra.

The cytochrome *c* content (mg of cytochrome *c* per g of dry cells) of *poky* is variable with age. During conidial germination (10–20 hr) the cytochrome *c* content increases rapidly (Figure 3) and reaches a maximum of 3% of the dry weight of the cell and then declines. This is in agreement with previous results of Haskins *et al.* (1953) which indicated that the *poky* phenotype is most extreme in young cultures. The specific activity of the purified ^{14}C-labeled cytochrome *c* follows the same general pattern. The values are high and subsequently decline.

From the total cytochrome *c* content and the ratio of C_I to C_{II} determined from the chromatographic elution profiles, a time course for the C_I and C_{II} content of *poky* was calculated (Figure 3). A sequential synthesis of the two proteins is evident. C_{II} constitutes all the cytochrome *c* during the initial 20 hr of growth, the interval of cytochrome *c* accumulation. After this time the amount of C_{II} decreases and C_I becomes the dominant cytochrome *c*. The C_{II} decrease parallels the decline of the cytochrome *c* content of *poky*, suggesting a relationship between C_{II} and the accumulation of cytochrome *c* in *poky*.

Both C_I and C_{II} were detected in *wild-type Neurospora* and the respiration-deficient mutants *mi-3*, *cty-1*, and *po-f* (Mitchell *et al.*, 1953; Mitchell, and Mitchell, 1956). This would indicate that the occurrence of two cytochrome *c*'s is not the result of the *poky* mutation. As shown in Figure 4 the ratio of C_I to C_{II} in these strains is similar to that of *wild type* at all ages, even though these mutants accumulate cytochrome *c*.

The temporal separation of the synthesis of C_{II} and C_I suggested a precursor–product relationship between the two proteins. This relationship was shown by following the kinetics of [^{14}C]lysine incorporation into C_{II} and C_I *in vivo* during pulses of varying lengths of time, by the *in vivo* conversion of C_{II} into C_I in the absence of appreciable protein synthesis using cycloheximide, and by pulse-labeling young *poky* with [^{14}C]lysine and demonstrating a quantitative transfer of ^{14}C from C_{II} to C_I. Only the latter experiment is described in detail.

Germinated *poky* conidia (13.3 g) were labeled with 15 μCi of [U-^{14}C]lysine in a pulse-chase experiment. The conidia were incubated for 30 min at 25° with the [^{14}C]lysine then transferred to fresh media containing 1000-fold excess of [^{12}C]lysine. This sample was divided between two carboys, each containing 16 l. of Vogel's medium N. One culture was collected after 10-hr growth and the second after 20 hr.

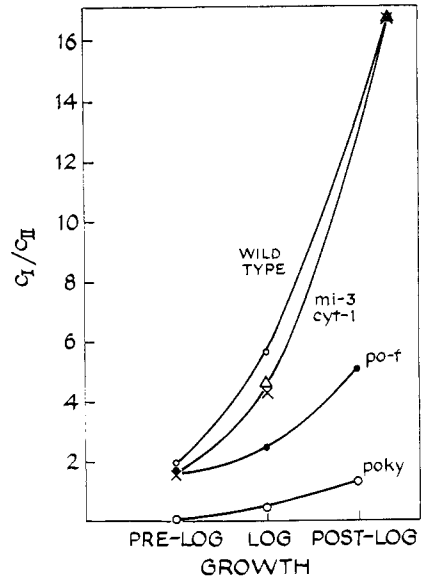

FIGURE 4: Ratio of C_I and C_{II} in various ages of *wild type* and several respiration-deficient strains of *Neurospora*. The ratios of C_{II} and C_I were determined from chromatographic elution profiles.

It was necessary to incubate the cultures for an additional 10 hr after treatment with [^{12}C]lysine since the chase was not always effective. By 10 hr no radioactivity was extractable from the free amino acid pool with cold trichloroacetic acid (Roberts *et al.*, 1955).

The cytochrome *c* extracted from the 10-hr culture was all C_{II}, while only C_I was detected in the 20-hr sample. The number of counts in C_{II} at 10 hr was equal to that found in C_I at 20 hr. During the same interval the specific activity of the cytochrome *c* decreased by 3.5-fold, which is comparable with the amount of cytochrome *c* synthesized (3.4-fold increase). This result is compatible with the idea that C_{II} is a precursor of C_I since the radioactivity originally present in C_{II} is quantitatively transferred to C_I.

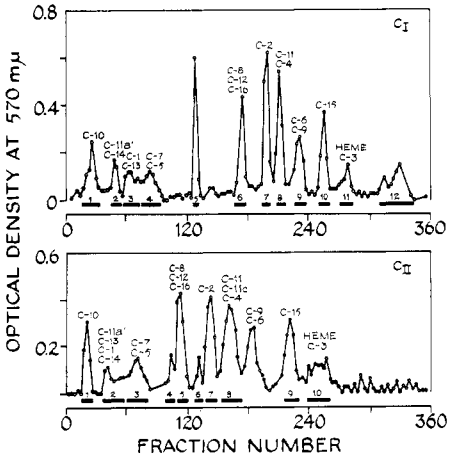

FIGURE 5: Peptide elution patterns from chymotryptic hydrolysates of C_I and C_{II}. The digest was chromatographed on a Dowex 50-X2 column with pyridine–acetate buffers as described in the text. The solid horizontal lines indicate fractions pooled. The numbers above the peaks refer to the peptides found in each particular pool. The nomenclature of the peptides is described in the text.

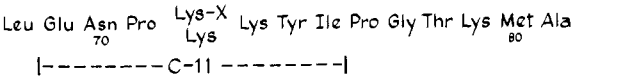

FIGURE 6: Partial amino acid sequence of *Neurospora* cytochrome *c* from residues 68 to 81 as determined by Heller and Smith (1966). Chymotryptic peptide C-11 is shown below the sequence as a dotted line. The position of Lys-X at residue 72 in C_{II} is indicated. The numbering of the residues is described by Margoliash and Schejter (1966).

Intracellular Distribution of C_I and C_{II}. To determine if C_I or C_{II} is preferentially bound to mitochondria *in vivo*, a sample of *poky* was divided in half. From one part mitochondria were isolated and purified by isopycnic centrifugation in sucrose gradients (Luck, 1963). Cytochrome *c* was extracted from the purified mitochondria with alkali at pH 10.5 and purified. From the second sample of *poky*, cytochrome *c* was extracted from the whole cell and purified. The ratio of C_{II} to C_I in the cytochrome *c* purified from the whole cell was 0.55. All the extractable cytochrome *c* from the mitochondria was C_I.

Structural Difference between C_I and C_{II}. Tryptic peptide maps revealed no significant differences between C_I and C_{II}. In addition total amino acid analyses of both proteins were similar and in good agreement with those previously determined for *Neurospora* cytochrome *c* (Heller and Smith, 1966).

The amino acid sequence of *Neurospora* cytochrome *c* has been determined by Heller and Smith (1966). The amino acid compositions of the chymotryptic peptides of C_I and C_{II} were compared with the results of these authors. Chymotryptic digests of C_I and C_{II} separated on Dowex 50 columns (Figure 5) were further purified by pH 3.5 high-voltage electrophoresis. All peptides except C-7 and C-13 were judged pure by amino acid analysis. The latter two peptides migrated together at pH 3.5 but were separable by preparative chromatography followed by pH 3.5 electrophoresis.

To rule out a difference in amide content of comparable C_I and C_{II} peptides, the electrophoretic mobilities of the peptides were compared at pH 1.9, 3.5, and 6.0. No significant mobility differences were detected. Amino acid analyses of the comparable C_I and C_{II} peptides revealed no differences except for peptides C-11 (see Figure 6). Although the electrophoretic mobilities of the comparable peptides C_I-11 and C_{II}-11 are similar, their amino acid compositions are not. As shown in Table I, peptides C_I-11 and C_{II}-11 differ in lysine content. Peptide C_{II}-11 contains 2 moles of lysine while peptide C_I-11 has only 1 mole of lysine and an additional basic amino acid with an odd electrophoretic mobility. This odd amino acid, designated as Lys-X, has a mobility between that of lysine and arginine (Figure 7) on pH 1.9 paper electrophoresis.

The two lysine residues of peptide C-11 occur at positions 72 and 73 of the cytochrome *c* molecule (Figure 6). A limited pronase digestion of peptide C_I-11 was carried out at 35° for 24 hr to determine the position of Lys-X. A fragment isolated by pH 3.5 paper electrophoresis had the composition (Asp, Lys-X). This result suggests that Lys-X occurs at residue 72 since asparagine and proline occur at residues 70 and

TABLE I: Amino Acid Compositions of Peptides C_I-11 and C_{II}-11.[a]

Amino Acid	Molar Ratio	
	Peptide C_I-11	Peptide C_{II}-11
Lysine	1.2 (1)[b]	1.7 (2)
Lys-X	1.0 (1)	0
Leucine	1.0 (1)	1.0 (1)
Glutamic acid	1.7 (2)	1.8 (2)
Asparatic acid and tyrosine	2.4 (3)	2.5 (3)

[a] The peptides were purified by pH 6.0 paper electrophoresis. Amino acid analysis was carried out by high-voltage paper electrophoresis and developed with the cadmium–ninhydrin stain. [b] Values in parentheses are the theoretical values taken from the amino acid sequence of *Neurospora* cytochrome *c* (Heller and Smith, 1966).

71, respectively. Proline was assumed to be present in the fragment although it was not detected because it develops as a pale yellow color with the cadmium–ninhydrin stain.

Lysine Labeling of Lys-X. The only structural difference between C_I and C_{II} is at residue 72 and the *in vitro* labeling experiments indicated a quantitative transfer of radioactivity from C_{II} to C_I. For these data to be compatible, the lysine at residue 72 in C_{II} must be converted into Lys-X in C_I. This is shown indirectly by demonstrating that Lys-X is a lysine derivative.

Poky (137 g) was incubated for 30 min with 1 mCi of DL-[4,5-^{3}H]lysine. Purified C_I isolated from this sample was hydrolyzed with chymotrypsin. The specific activity of amino acids from each purified chymotryptic peptide was estimated. The amount of radioactivity in all 18 different amino acids except that of tryptophan and cysteine was determined. Only lysine and Lys-X had radioactivity significantly above background, indicating that the lysine label had not randomized. The results for peptide C_I-11 are given in Table II. The average specific activity of lysine and Lys-X was 5.3, while the values of all other amino acids was less than 0.6. Since the lysine and Lys-X specific activities are comparable, it was concluded that Lys-X is a lysine derivative In addition Lys-X has an electrophoretic mobility similar to that of several lysine derivatives (Figure 8), which is compatible with the results of DeLange *et al.* (1969) who found that residue 72 in *Neurospora* cytochrome *c* is ϵ-trimethyllysine.

TABLE II: Distribution of [^{3}H]Lysine among the Amino Acids of Peptide C_I-11.[a]

Amino Acid	cpm	mμmoles	Sp Act. (cpm/mμmole)
Lysine	345	59.5	5.8
Lys-X	307	57.8	5.3
Leucine	4.8	62.4	0.08
Glutamic acid	3.0	125.2	0.02
Aspartic acid + tyrosine	7.4	137.8	0.02

[a] Peptide C_I-11 was purified by pH 6.0 electrophoresis. Amino acids were separated by pH 1.9 electrophoresis and were stained with cadmium-ninhydrin. Radioactivity was determined in the methanol extract of the paper and the paper itself after extraction. Approximately 30% of the stain and lysine radioactivity was eluted from the paper by this method. No radioactivity was detected for any of the other amino acids in the extracted paper. The number of counts reported are that amount in the methanol extract.

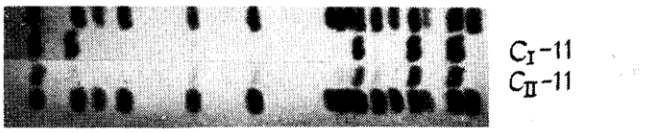

FIGURE 7: Qualitative amino acid compositions of peptides C_I-11 and C_{II}-11. Both peptides were purified by electrophoresis at pH 6.0. The amino acids were separated by pH 1.9 paper electrophoresis after acid hydrolysis of the peptides. The papers were stained with the cadmium-ninhydrin reagent. The anode is on the right, the cathode on the left. Standard mixtures of amino acids were run alongside of each sample. From left to right, the amino acids in the standard are Lys, Arg, His, aminoethyl-Cys, Gly, Ala, Val, Ser, Ile, Leu, Thr, Met, Glu, Phe, (Asp, Tyr), and Trp. Lys-X in peptide C_I-11 has a mobility between Lys and Arg.

Discussion

These results confirm the earlier suggestion of Hardesty (1961) that the *Neurospora* cytochrome *c* is heterogeneous. Two chromatographically separable cytochrome *c*'s, C_{II} and C_I, were detected in the *poky* and *wild-type* strains. The temporal separation of C_I and C_{II} synthesis in *poky* (Figure 3) would explain the discrepancy between these results and those of Heller and Smith (1966). These authors extracted cytochrome *c* from *poky* that was physiologically quite old and therefore detected only one cytochrome *c*, C_I. The two cytochrome *c*'s are not artifacts of the harsh extraction conditions as are most multiple chromatographic forms of cytochrome *c*. Both cytochrome c's have similar sedimentation coefficients (Figure 2), are stable chromatographic species, and have identical ultraviolet and visible absorption spectra. These facts eliminate the possibility that the chromatographic

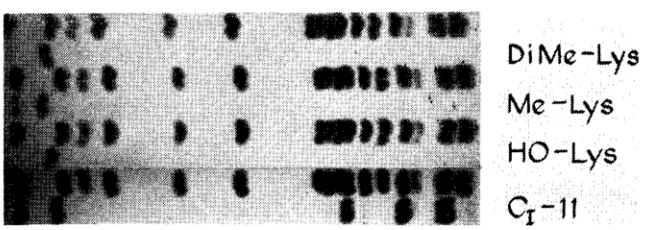

FIGURE 8: Electrophoretic mobility of hydroxylysine (HO-Lys), ϵ-*N*-methyllysine (Me-Lys), ϵ-*N*-dimethyllysine (DiMe-Lys), and Lys-X (in peptide C_I-11). Electrophoresis was carried out at pH 1.9 under the same conditions as in Figure 7. The unmarked samples are the standard mixture of amino acids. The order of each amino acid is described in Figure 7.

separation of C_I and C_{II} is an artifact due to polymerization, aggregation with basic proteins, or differences of oxidation state. This conclusion is also supported by the observation that both C_I and C_{II} are detected in buffer (0.05 M Tris-HCl, pH 8.6) extracted cytochrome *c* preparations.

The time-course measurements of cytochrome *c* in *poky* indicate a temporal separation of C_I and C_{II} synthesis (Figure 3). C_{II} is synthesized first, followed by the synthesis of C_I and the concomitant disappearance of C_{II}. The temporal separation of C_{II} and C_I synthesis is reminiscent of the oxygen-induced synthesis of the yeast isocytochrome *c*'s (Slonimski *et al.*, 1965). In both organisms the cytochrome *c* that adsorbed more strongly on ion-exchange resins is synthesized first. However, the yeast isocytochrome *c*'s are encoded by separate structural genes (Sherman *et al.*, 1966) and are known to differ by 13 amino acid residues (Stewart *et al.*, 1966). In *Neurospora*, the time-course curves of C_{II} and C_I synthesis resemble those for a precursor and product. The pulse-chase experiments with radioactive lysine also support this relationship. Radioactivity initially incorporated into C_{II} *in vivo* is quantitatively transferred to C_I while the cytochrome *c* complement of the cell changes from C_{II} to C_I. Therefore, the synthesis of two cytochrome *c*'s in yeast and *Neurospora* are not comparable situations.

C_{II} and C_I differ in amino acid sequence at residue 72. In C_{II} this residue is lysine (Table I). The equivalent residue in C_I, a basic amino acid designated as Lys-X, has an electrophoretic mobility between lysine and arginine (Figure 7). If C_I and C_{II} are encoded by the same structural gene, Lys-X must be a lysine derivative. This is supported by the data of Table II. The specific activities of Lys-X and the other lysine residues of C_I labeled with [4,5-^{3}H]lysine are identical. In addition, Lys-X has an electrophoretic mobility similar to that of several lysine derivatives (Figure 8). These results are compatible with the finding of ε-trimethyllysine at residue 72 in *Neurospora* cytochrome *c* (DeLange *et al.*, 1969). C_{II} is undoubtedly converted into C_I by a specific methylation of lysine-72 in C_{II} to give ε-trimethyllysine in C_I.

The position of ε-trimethyllysine in the amino acid sequence is interesting. The amino acid sequence from residues 70 to 80 of cytochrome *c* has been strictly conserved throughout the whole phylogenetic scale from molds to humans (Margoliash and Schejter, 1966). Margoliash (1966) has postulated that this indicates an integration of this region (residues 70–80) with a structure that has also remained invariant throughout evolution. Chemical modification studies have implicated either Lys-72 or -73 as a participant in the cytochrome oxidase reaction (Okunuki *et al.*, 1965) and Met-80 as an iron ligand of the heme moiety (Ando *et al.*, 1965). Both proposals are compatible with the 4-Å electron density map of horse heart cytochrome *c* obtained by Dickerson *et al.* (1967). An extended polypeptide chain bearing the sixth ligand was shown to run parallel to the long axis of the molecule and perpendicular to the plane of the heme. The ligand could easily be the methionine at residue 80, and residues 70–80 could be the extended polypeptide chain.

The accumulation of cytochrome *c* in young *poky* and its subsequent disappearance in older cultures is novel. The cytochrome *c* accumulation reaches a maximum at 20 hr with the growth conditions employed in these experiments (Figure 3). The decline of the cytochrome *c* accumulation is gradual over a period of 40–50 hr. This decline is apparently due to a decreased rate of cytochrome *c* synthesis rather than degradation, since the specific activities of the cytochrome *c* at different ages parallel the cytochrome *c* content of the cell (Figure 3). This idea is supported by the observation that radioactivity initially present in C_{II} is quantitatively transferred to C_I *in vivo* as the culture ages.

The existence of two cytochrome *c*'s and the temporal separation of their synthesis is not unique to the *poky* mutant. Both proteins were detected in *wild-type* and other respiration-deficient strains. In these latter strains, however, C_{II} is a minor component of the cytochrome *c* population during the earliest stages of growth (Figure 4). In contrast, cytochrome *c* accumulates in young *poky* as C_{II} until C_I is synthesized. Young *poky* apparently cannot affect the conversion of C_{II} into C_I during conidial germination. The importance of the conversion of C_{II} into C_I is indicated by the accumulation of cytochrome *c* as C_{II}.

The conversion of C_{II} into C_I may reflect the binding of the cytochrome *c* to the mitochondrial matrix. Only C_I was detected in purified mitochondria from young *poky* although C_I and C_{II} were present in a ratio of 0.55 in cytochrome *c* extracted from the whole cell. However, the possibility that C_{II} is not bound as tightly as C_I and leaches out of the mitochondria during purification has not been eliminated.

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Effect of Aminoacyl Transfer Ribonucleic Acid on Competition between Guanosine 5′-Triphosphate and Guanosine 5′-Diphosphate for Binding to a Polypeptide Chain Elongation Factor from *Escherichia coli**

David Cooper† and Julian Gordon

ABSTRACT: The polypeptide chain elongation factor preparation referred to as T (an undissociated combination of T_u and T_s) exhibited preferential binding of guanosine 5′-diphosphate, even in the presence of a large excess of guanosine 5′-triphosphate, when tested for retention on Millipore filters. Previously published data on binding of guanosine 5′-triphosphate using this assay is now shown to be attributable to the preferential binding of the [^{3}H]guanosine 5′-diphosphate present in the [^{3}H]guanosine 5′-triphosphate preparation. Removal of this guanosine 5′-diphosphate with a guanosine 5′-triphosphate regenerating system considerably reduced the level of bound nucleoside phosphate. Nonradioactive guanosine 5′-triphosphate did not compete with Millipore-bindable [^{3}H]guanosine 5′-diphosphate. Addition of aminoacyl transfer ribonucleic acid reversed this preference for guanosine 5′-diphosphate.

It is now clear that the mechanism by which aminoacyl-tRNA binds to *Escherichia coli* ribosomes during polypeptide synthesis involves the polypeptide chain elongation factor T of Nishizuka and Lipmann (1966), which was subsequently resolved into two factors, T_u and T_s, by Lucas-Lenard and Lipmann (1966). This binding also requires GTP (Ravel *et al.*, 1967, 1968; Lucas-Lenard and Haenni, 1968; Ertel *et al.*, 1968b). The binding of aminoacyl-tRNA to ribosomes was originally proposed to proceed in two steps, on the basis of comparison of Millipore binding assays, which detected the first step (T–GTP); and gel filtration assays with Sephadex G-50 which detected a ternary complex (T–GTP–aminoacyl-tRNA), characterized by its inability to bind to the Millipore (Gordon, 1967, 1968; Ravel *et al.*, 1968; Ertel *et al.*, 1968a). More recent studies have suggested that the subfraction T_u is the acceptor for nucleoside phosphate (Ertel *et al.*, 1968a; Ravel *et al.*, 1969). However, in the experiments described in this paper, T_u and T_s were not dealt with separately, so we retain the terminology T factor for convenience.

More detailed experiments have now shown that the Millipore assay selectively detects the binding of the trace of GDP preexisting in commercial preparations of GTP, and this preference was overcome by the addition of aminoacyl-tRNA. These experiments are the subject of this communication.

Materials and Methods

GTP was supplied by P-L Biochemicals, Inc., and repurified essentially by the method of Moffatt (1964). A 0–0.4 M triethylammonium bicarbonate gradient (pH 7.5) was used to elute the nucleotides from a DEAE-cellulose column (Whatman DE-11). Fractions containing GTP were identified by thin-layer chromatography as described below, pooled, and lyophilized.

[^{3}H]GTP (lithium salt, specific activity 1.4 Ci/mmole) and [^{3}H]GDP (lithium salt, specific activity 1.27 Ci/mmole) were

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