A cytochrome *c* methyltransferase from *Crithidia oncopelti*

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The mitochondrial cytochrome *c*-557 of *Crithidia oncopelti* contains two lysine residues and an *N*-terminal proline residue that are methylated *in vivo* by the methyl group of methionine. The purified cytochrome can act as a methyl acceptor for a methyltransferase activity in the cell extract that uses *S*-adenosylmethionine as methyl donor. *Crithidia* cytochrome *c*-557 is by far the best substrate for this methyltransferase of those tested, in spite of the fact that methylation sites are already almost fully occupied. The radioactive uptake of [¹⁴C]methyl groups from *S*-adenosylmethionine occurred only at a lysine residue (–8) and the *N*-terminal proline residue. This methyltransferase appears to differ from that of *Neurospora* and yeast [Durban, Nochumson, Kim, Paik & Chan (1978) *J. Biol. Chem.* **253**, 1427–1435; DiMaria, Polastro, DeLange, Kim & Paik (1979) *J. Biol. Chem.* **254**, 4645–4652] in that lysine-72 of horse cytochrome *c* is a poor acceptor. Also, the *Crithidia* methyltransferase appears to be able to carry lysine methylation much further to completion than do the enzymes from yeast and *Neurospora*, which produce very low degrees of methylation in native cytochromes *c*.

Methylation of proteins is a widespread phenomenon (Paik & Kim, 1980*a,b*), but the specificity of methylation and its functional significance are very poorly understood. Durban *et al.* (1978) isolated a highly specific cytochrome *c* methyltransferase from *Neurospora*, and a similar enzyme was subsequently shown to be present in yeast (DiMaria *et al.*, 1979). Although these enzymes are indeed most active with horse cytochrome *c* as substrate, only 0.2% of the cytochrome becomes methylated (Polastro *et al.*, 1978), and the reason for this is not known.

Cytochrome *c*-557 from the protozoan *Crithidia oncopelti* contains two residues of trimethyl-lysine (Pettigrew *et al.*, 1975), and the *N*-terminal proline is also methylated at the α -imino group (Pettigrew & Smith, 1977). Because of this extensive methylation we propose that *Crithidia oncopelti* might be a suitable source for a cytochrome *c*-specific methyltransferase. The present investigation seeks to characterize such an activity and to determine whether the transfer of methyl groups to proline and lysine residues requires the coenzyme *S*-adenosylmethionine as methyl donor and involves one or more than one enzyme.

Abbreviation used: Dns, 5-dimethylaminonaphthalene-1-sulphonyl.

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Methods

Growth of Crithidia oncopelti

Crithidia oncopelti, obtained from Dr. W. Gutteridge (University of Kent, Canterbury, U.K.), was grown as described by Pettigrew *et al.* (1975).

For growth in the presence of [¹⁴C]methionine, 12.5 μ Ci (0.2 μ mol) of the radioactively labelled amino acid (The Radiochemical Centre, Amersham, Bucks., U.K.) was added via a sterile filter (Swinnex, 0.22 μ m, Millipore) to each litre of autoclaved medium. Cells collected after 3 days' growth had taken up 64% of the radioactivity in the medium.

Purification of cytochrome c-557

Cytochrome *c*-557 was purified as described by Pettigrew *et al.* (1975). From 60 g of cells, approx. 1 μ mol of pure cytochrome *c*-557 was obtained ($A_{557, \text{red.}}/A_{280, \text{ox.}}$ ratio = 1). Small amounts of cytochrome lacking the *N*-terminal five amino acid residues (Pettigrew *et al.*, 1975) were also detected during purification, and this species, known as cytochrome *c*-557 (–5, 102) was also purified.

Amino acid and peptide separation and detection of radioactivity

(i) Amino acids obtained after total acid hydrolysis of [¹⁴C]methyl-labelled protein (6M-HCl at

105°C for 20h *in vacuo*) were dissolved in 0.1M-NH₃ and applied to Whatman no. 1 paper at a loading of 5–50nmol/cm. After electrophoresis in pH2.0 buffer (Ambler, 1963), the paper was cut into 1cm-wide strips and placed in scintillation fluid [toluene/Triton X-100 (2:1, v/v) containing 0.27% 2,5-diphenyloxazole and 0.007% 1,4-bis-(5-phenyloxazol-2-yl)benzene]. Guide strips of standard amino acid mixtures were run in parallel and stained with ninhydrin (0.2%, w/v, in acetone).

(ii) Quantitative amino acid analysis was performed by the general method of Spackman (1963) with a Locarte amino acid analyser. For separation of the various methyl-lysines a modified third buffer was used that contained 0.45% boric acid, 0.5% trisodium citrate and 0.15% NaOH (all w/v). This allowed separation of the three methyl-lysines and lysine in 100 min on a 20cm column of resin (Fig. 1). Changing the trisodium citrate concentration to 0.4% resulted in a better separation of trimethyl-lysine from lysine and was used in some cases. Although the pH of this buffer is 9.5, it is only weakly buffering, and its addition to the ninhydrin solution does not greatly affect the pH of the reaction mixture. The colour yield for trimethyl-lysine was assumed to be identical with that for arginine (DeLange *et al.*, 1969).

(iii) Peptides obtained from tryptic digestion of cytochrome *c*-557 were purified as described by Pettigrew *et al.* (1975). The blocked *N*-terminal tryptic peptide was located on paper either by arginine fluorescence after spraying with phenanthrenequinone (Yamada & Itano, 1966) or by spraying the paper with 0.04% Thymol Blue in ethanol/butan-1-ol (1:1, v/v) containing 3mM-H₂SO₄ (Ingram, 1953). Other peptides were located by dipping in ninhydrin solution (0.2%, w/v, in acetone).

(iv) With the *N*-terminal pentapeptide of *Crithidia* cytochrome *c*-557 the location of [¹⁴C]methyl label was investigated by total acid hydrolysis followed by treatment with dansyl chloride (Dns-Cl) (Gray, 1972), and separation of Dns-amino acids on polyamide sheets (Ramshaw *et al.*, 1970). Fluorescent spots were cut out and placed in scintillation liquid for counting of radioactivity.

Gel electrophoresis of proteins and detection of radioactivity

The method of Anker (1970) was used. Gel cylinders (0.5cm × 8cm) were 15% (w/v) in acrylamide and 0.4% (w/v) in *NN'*-diallyltartardiamide, and were made up in 50mM-sodium acetate buffer, pH5.0. The gels were pre-run for 1h before cytochrome samples were loaded on (approx. 2nmol/gel). After electrophoresis at 4mA/gel for 4h, the gels were scanned at 410nm (Gilford model 222A photometer) and then cut into 0.25cm slices.

Fig. 1. Methyl-lysines in *Crithidia* cytochrome *c*-557. The trace is part of an amino acid analysis of an acid hydrolysate of *Crithidia* cytochrome *c*-557 with the modified third buffer described in the Methods section to separate the methyl-lysines. Calculation of the quantities present gives the following values (mol/mol of cytochrome *c*-557); lysine, 10; mono-methyl-lysine, trace; dimethyl-lysine, 0.033; trimethyl-lysine, 1.98.

Each slice was incubated in 0.5ml of 4% (w/v) periodic acid at 55°C until it dissolved. Then 0.2ml of 2M-glucose was added and finally 10ml of scintillation liquid before liquid-scintillation counting of radioactivity.

Tryptic digestion

For complete tryptic digestion, apocytochrome (Pettigrew *et al.*, 1975) was treated with 0.025% (w/w) trypsin (diphenylcarbamoyl chloride-treated; Sigma Chemical Co.) in 0.1M-ammonium acetate buffer, pH8.5, at 37°C for 4h. The digestion mixture was freeze-dried.

For limited tryptic digestion, the intact cytochrome was treated with 0.002% (w/w) trypsin (diphenylcarbamoyl chloride-treated) in 5 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.0, at 37°C for 1 h and the reaction was stopped by the addition of a 2-fold molar excess (over trypsin) of soya-bean trypsin inhibitor (Sigma Chemical Co.).

Enzyme assay

This was a modification of the method of Durban *et al.* (1978). The reaction was performed in 15 ml conical centrifuge tubes, in a final volume of 0.5 ml. The reaction mixture contained 0.1 M-glycine/NaOH buffer, pH 9.0, 0.4 mM-EDTA, 5.7 mM-2-mercaptoethanol, 5 nmol of cytochrome *c*, 2 mg of crude enzyme, 0.1 μCi (1.7 nmol) of *S*-adenosyl-L-[^{14}C]methionine (from a 57 $\mu\text{Ci}/\mu\text{mol}$ 10 $\mu\text{Ci}/\text{ml}$ stock solution in 1 mM- H_2SO_4 stored at -40°C).

The solution was incubated at 37°C for 5 min before the addition of enzyme and *S*-adenosyl-[^{14}C]methionine. The reaction was then allowed to proceed at 37°C for 30 min and was terminated by the addition of 0.5 ml of a 1:1 (v/v) slurry of CM-cellulose (Whatman CM-52) in 5 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.0. The cytochrome was adsorbed on the CM-cellulose, and was washed twice with 5 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.0, and then eluted with 1 ml of the same buffer containing 5 M-NaCl. Eluted proteins were precipitated by addition of an equal volume of 15% (w/v) trichloroacetic acid and then treated as described by Durban *et al.* (1978). The final pellet was decolorized by using the method of Neame (1977) and placed into scintillant for counting of radioactivity.

Preparation of crude methyltransferase

All operations were performed at 0–4°C. Approx. 1.0 g of stored frozen *Crithidia* was thawed and refrozen twice. The procedure resulted in complete disruption of the cells as determined by microscopic examination. The cells were homogenized in 2 vol. of 5 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.0, in a ground-glass homogenizer and then centrifuged in a Beckman J-21 centrifuge at 17000 g for 30 min. The supernatant was made 30% saturated with solid $(\text{NH}_4)_2\text{SO}_4$, without adjustment of pH, and stirred on ice for 15 min, and then centrifuged at 17000 g for 45 min.

The resulting supernatant was made 50% saturated with solid $(\text{NH}_4)_2\text{SO}_4$ and treated as above. The supernatant was discarded, and the pellet was dissolved in a minimum volume (approx. 1–1.5 ml) of 5 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.0, and dialysed against this buffer for 2 h. The protein concentration of the dialysis residue was determined by the method of Schacterle & Pollack (1973), and the crude enzyme was stored at -40°C. Enzyme activity remained stable for 10 days.

Results

Incorporation of [^{14}C]methyl groups from [^{14}C]methionine into Crithidia cytochrome c-557

Cytochrome *c*-557 (0.6 μmol) was isolated from cells grown in the presence of [^{14}C]methionine. The specific radioactivity of the purified cytochrome was 1.12×10^5 c.p.m./ μmol . The haem group was removed and the apoprotein was digested with trypsin. Peptides, 'mapped' and isolated as described by Pettigrew *et al.* (1975), were analysed for amino acids, and radioactivity was determined by scintillation counting. The specific radioactivities of selected peptides are shown in Table 1. When these specific radioactivities were recalculated on the basis of methyl groups, the values shown in the last column were obtained, which covered the small range 1.33×10^4 – 1.56×10^4 c.p.m./ μmol of methyl group for all those peptides that contained methyl groups derived from methionine.

The *N*-terminal tryptic peptide (T1), containing both dimethylproline and trimethyl-lysine, was hydrolysed and the constituent amino acids were separated by electrophoresis at pH 2 and located by using the Thymol Blue indicator spray. After elution of the peptides from the paper, portions of each amino acid were taken for amino acid analysis and scintillation counting of radioactivity, and the specific radioactivities are included in Table 1. For dimethylproline it is not possible to determine quantity by amino acid analysis, and in this case it was assumed that an amount equal to that of proline was recovered from the paper.

Development of the assay method

At the start of the present investigation, the assay method of Durban *et al.* (1978), with horse cytochrome *c* as substrate, was used. In this method the [^{14}C]methylated horse cytochrome *c* was precipitated with trichloroacetic acid. When this assay was applied to *Crithidia* cell extracts, large amounts of radioactivity were found associated with the trichloroacetic acid precipitates, but this was so in the absence as well as in the presence of horse cytochrome *c*.

We concluded that extensive methylation of endogenous proteins was occurring and obscuring any methylation of the horse cytochrome *c* substrate. To overcome this problem, we stopped the reaction by the addition of CM-cellulose to adsorb the horse cytochrome *c*, and then eluted the cytochrome from the CM-cellulose, precipitated it with trichloroacetic acid and measured the radioactivity in the precipitate. Again we observed no difference between assay mixtures containing horse cytochrome *c* and those without, and again large amounts of radioactivity were associated with proteins adsorbed on CM-cellulose.

Table 1. *Incorporation of [¹⁴C]methyl groups into Crithidia cytochrome c-557 in vivo*

Peptides from [¹⁴C]methylated cytochrome c-557 (specific radioactivity 1.1×10^5 c.p.m./ μ mol) were isolated as described in the Methods section. Portions of each were used for amino acid analysis and liquid-scintillation counting of radioactivity. Radioactivities of vials were counted for 1 min and samples contained more than 500 c.p.m. above a background of 25 c.p.m. Counter efficiency was 90%. The peptide nomenclature is that of Pettigrew *et al.* (1975), and the amino acid sequence positions of residues that contain radioactive methyl groups are shown. Peptide T12 is included as an example of a peptide not containing a methyl group from methionine, and was unlabelled. The radioactivity associated with proline derived from peptide T1 is probably due to incomplete separation from dimethylproline.

Peptide	Sequence position	Methylated amino acid	Sp. radioactivity (c.p.m./ μ mol of peptide)	Methyl groups derived from methionine	Sp. radioactivity (c.p.m./ μ mol of methyl group)
T9	Ala-51-Lys-73	Me ₃ Lys-72	4.5×10^4	3	1.5×10^4
T10	Phe-74-Lys-79	Met-75	1.4×10^4	1	1.4×10^4
T11	Met-80-Lys-86	Met-80	1.5×10^4	1	1.5×10^4
T12	Lys-87-Arg-91	—	0	—	—
T1	Me ₂ Pro—10-Arg—6	Me ₂ Pro—10 Me ₃ Lys—8	6.8×10^4	5	1.4×10^4
Amino acids from peptide T1		Me ₂ Pro	3×10^4	2	1.5×10^4
		Me ₃ Lys + Arg	4×10^4	3	1.3×10^4
		Ala	0	—	—
		Pro	(6×10^2)	—	—

Gel electrophoresis of *Crithidia* proteins that could be adsorbed on CM-cellulose indicated that such basic proteins were few in number and included *Crithidia* cytochrome c-557. In order to remove this potential endogenous substrate from cell extracts, (NH₄)₂SO₄ fractionation was performed, and it was found that the precipitate collected between 30 and 50% saturation contained 85% of the methyltransferase activity but no *Crithidia* cytochrome c-557.

The final assay conditions used throughout the present work therefore involved preparation of the methyltransferase activity by (NH₄)₂SO₄ fractionation and adsorption of the labelled substrate after reaction on CM-cellulose as described in the Methods section.

The incorporation of [¹⁴C]methyl groups into the substrate was stable to treatment with alkali and therefore not due to methylation of arginine or formation of *O*-methyl esters (Paik & Kim, 1980a,b). The incorporation of label was enzymically mediated, since boiling destroyed activity.

Reactivity of cytochromes with Crithidia cytochrome methyltransferase

With this assay *Crithidia* cytochrome c-557 was by far the best substrate examined (172 c.p.m. incorporated/nmol of cytochrome), in spite of the fact that amino acid sequence information demonstrated that, unlike horse cytochrome c, *Crithidia* cytochrome c-557 is already methylated (Pettigrew *et al.*, 1975; Smith & Pettigrew, 1980; Fig. 2).

However, when cytochrome c-557 (—5, 102) was used as substrate very little incorporation of radioactivity was detected, indicating that almost all the radioactivity entering the intact cytochrome c-557 may be associated with the *N*-terminal five amino acid residues. Under the same assay conditions, horse heart, baker's yeast and *Candida crusei* cytochromes c each show incorporations of less than 6 c.p.m./nmol of cytochrome.

Examination of the amino acid analysis of *Crithidia* cytochrome c-557 revealed the presence of small amounts of mono- and di-methyl-lysine (Fig. 1). Pettigrew *et al.* (1975) had shown that lysine—8 was not fully methylated, unlike lysine-72. It therefore seemed likely that lysine—8 was acting as acceptor for methyl transfer from *S*-adenosyl-methionine in the assay of *Crithidia* cytochrome c-557.

From amino acid analysis of cytochrome c-557, this methyl-accepting ability, shown as 'available sites' in Table 2, is only 0.033 mol/mol of cytochrome (Fig. 1). For horse cytochrome c, lysine-72 is completely unmethylated in the natural state, and therefore, if [¹⁴C]methyl groups predominantly enter this site (Durban *et al.*, 1978), this cytochrome has 3 mol of available sites/mol of protein. In Table 2 calculations are made on this basis of the efficiency of the methyltransferase reactions in *Neurospora* and *Crithidia* extracts, and the results show that *Crithidia* methyltransferase methylates *Crithidia* cytochrome c-557 about 200 times more efficiently than does *Neurospora* methyltransferase with horse cytochrome c.

Fig. 2. *Methylation in mitochondrial cytochromes c*

All mitochondrial cytochromes *c* can be aligned without the use of gaps to give greater than 40% identity between pairs of sequences. This Figure is a summary of such alignments with emphasis on the variable *N*-terminal region and the methylated amino acids. Vertebrates and plants have acetylated cytochromes *c*, and plant cytochromes *c* have an eight-residue extension at the *N*-terminus. Fungi and yeasts have cytochromes *c* with a small *N*-terminal extension of four to eight residues retaining a free amino group (Dickerson & Timkovich, 1975). The methylation pattern in yeasts and fungi is more complex than indicated here, since *Humicola* cytochrome *c* has dimethyl-lysine at position 72 and trimethyl-lysine at position 86.

Table 2. Degree of methylation catalysed by *Neurospora* and *Crithidia* methyltransferases

Values used for mol of methyl group incorporated/mol of protein were in each case the best ones obtained. The available sites were calculated on the assumption that *Neurospora* methylase acts specifically at lysine-72 of horse heart cytochrome *c* (Durban *et al.*, 1978), which is isolated in the fully unmethylated state and therefore can accept 3 mol of methyl group/mol. The value for the available sites in *Crithidia* cytochrome *c*-557 was calculated from the amino acid analysis (Fig. 1), which showed 0.033 mol of dimethyl-lysine/mol and only a trace of monomethyl-lysine. This value may be a slight underestimate if any unmethylated lysine is present at position -8. The value of 0.07% completion for the *Neurospora* enzyme with horse heart cytochrome *c* is derived from the value of 0.2% methylation given by Durban *et al.* (1978, Miniprint Fig. 6S) divided by 3 to give percentage methylation on the basis of 3 mol of methyl-accepting sites/mol of horse heart cytochrome *c*.

Source of enzyme	Cytochrome <i>c</i>	Available sites (no./molecule)	Methyl group incorporated (mol/mol of protein)	% completion of reaction
<i>Neurospora</i>	Horse	3	0.002	0.07
<i>Crithidia</i>	Horse	3	0.00002	0.001
<i>Crithidia</i>	<i>Crithidia</i>	0.033	0.0046	14

Preparation of [^{14}C]methyl-labelled *Crithidia* cytochrome *c*-557

A 15 nmol sample of *Crithidia* cytochrome *c*-557 was treated with 1.8 mg of crude enzyme and 0.5 μCi of *S*-adenosyl[^{14}C]methionine under otherwise standard assay conditions. The reaction was stopped by the addition of 1 ml of cold 5 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.2, and the solution was passed through a column (0.3 cm diam. $\times$ 2 cm) of CM-cellulose equilibrated in the same buffer. The column was washed with 2 column volumes of the buffer, and the cytochrome was eluted with the buffer containing 0.5 M-NaCl. The cytochrome solution was passed through a Sephadex G-25 (fine grade) column (0.3 cm diam. $\times$ 7 cm) equilibrated with 5 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.0.

Electrophoresis in polyacrylamide gels in 50 mM-sodium acetate buffer, pH 5.0, indicated that all the radioactivity was associated with the cytochrome and that the enzymically methylated cytochrome was indistinguishable from unmodified cytochrome in the electrophoresis system used.

Amino acid residues in *Crithidia* cytochrome *c*-557 that accept [^{14}C]methyl groups

A portion of labelled cytochrome (10 nmol) was hydrolysed in 6 M-HCl, and the amino acids were separated by paper electrophoresis at pH 2. Most (approx. 95%) of the radioactivity was found at a mobility corresponding to arginine and trimethyl-lysine, and a small amount (approx. 5%) at the mobility of dimethylproline (Fig. 3).

Location of the methylation sites in the sequence of *Crithidia* cytochrome *c*-557

As discussed above, the *N*-terminal pentapeptide of *Crithidia* cytochrome *c*-557 is the probable location of [^{14}C]methylation. During purification of

Crithidia cytochrome *c*-557 a minor species was isolated, in variable amount, that lacked the *N*-terminal pentapeptide. This species, cytochrome *c*-557 (-5, 102), could be produced *in vitro* by limited tryptic digestion of the holocytochrome *c*.

Limited tryptic digestion of *Crithidia* cytochrome *c*-557

It was found that optimum conditions for producing this truncated cytochrome by tryptic digestion were a 1:500 (w/w) trypsin/cytochrome *c*-557 ratio at 37°C for 1 h. Under these conditions little hydrolysis occurs at sites other than Arg-6-Glu-5.

When such a reaction mixture was separated by gel electrophoresis and the gel was scanned at 410 nm and then sliced and counted for radioactivity, the pattern shown in Fig. 4 was obtained. No radioactivity was found at the position of the truncated cytochrome *c*-557 (-5, 102). Apart from undigested cytochrome, the only other radioactive peak was one of high mobility, which is proposed to be the basic *N*-terminal pentapeptide. To confirm these identifications, the experiment was repeated on a larger scale with the digestion of 107 nmol of *Crithidia* cytochrome *c*-557 that had been [^{14}C]methylated under standard reaction conditions. After the reaction had been stopped by the addition of 2 mol of soya-bean trypsin inhibitor/mol of trypsin, the digestion mixture was passed through a Sephadex G-25 (fine grade) column (1.5 cm diam. $\times$ 15 cm) equilibrated in 10 mM-ammonium acetate buffer, pH 5.0. The absorbance at 410 nm and the radioactivity content of the fractions collected were measured. The cytochrome fractions contained a small amount of radioactivity, but most (87%) was eluted after the cytochrome, consistent with it being of low molecular weight. The two fractions were analysed separately.

Fig. 3. Radioactivity labelled amino acids in *Crithidia* cytochrome *c*-557 after reaction with *Crithidia* methyltransferase and *S*-adenosyl[Me-¹⁴C]methionine

Crithidia cytochrome *c*-557 (10 nmol) was incubated under the standard reaction conditions with *S*-adenosyl[Me-¹⁴C]methionine (0.15 μ Ci) and crude *Crithidia* methyltransferase (2 mg). The cytochrome was recovered from the reaction mixture as described in the Methods section and then hydrolysed in 6 M-HCl at 110°C for 24 h. The dried hydrolysate was applied to paper, and the amino acids were separated by electrophoresis at pH 2.0 at 5 kV for 20 min. Strips, 1 cm wide, were placed in scintillation fluid for counting of radioactivity. The positions of standard amino acids were also determined. Key: K, Lys; R, Arg; G, Gly; A, Ala; V, Val; S, Ser; T, Thr; E, Glu; D, Asp; H, His; L, Leu; P, Pro; F, Phe; Y, Tyr.

Analysis of species produced by limited tryptic digestion of *Crithidia* cytochrome *c*-557

The cytochrome solution was diluted with 1 vol. of water and passed through a CM-cellulose column (1.5 cm diam. $\times$ 8 cm) equilibrated in 5 mM-NaH₂PO₄/Na₂HPO₄ buffer, pH 7.0. The CM-cellulose was washed with 3 vol. of this buffer and then with 30 mM-NaH₂PO₄/Na₂HPO₄ buffer, pH 7.0. Most of the cytochrome (fraction 1) was eluted

Fig. 4. Polyacrylamide-gel electrophoresis of [¹⁴C]-methyl-labelled *Crithidia* cytochrome *c*-557 after partial tryptic digestion

Polyacrylamide-gel electrophoresis under the conditions described in the Methods section was performed on [¹⁴C]methyl-labelled *Crithidia* cytochrome *c*-557 that had been partially digested with trypsin (1:500, w/w). The gel was scanned at 410 nm and then sliced for solubilization and liquid-scintillation counting of radioactivity.

under these conditions. A small amount of cytochrome (fraction 2) remained attached to the column and was eluted with 5 mM-NaH₂PO₄/Na₂HPO₄ buffer, pH 7.0, containing 0.5 M-NaCl.

Portions of these two cytochrome solutions were examined by amino acid analysis, *N*-terminal analysis, associated radioactivity and behaviour on CM-cellulose. The results were consistent with fraction 1 being the truncated cytochrome *c* (–5, 102) and fraction 2 being intact cytochrome, all the radioactivity being associated with the latter fraction.

Analysis of peptides produced by limited tryptic digestion of *Crithidia* cytochrome *c*-557

The radioactive low-molecular-weight fraction obtained by gel filtration of the tryptic digest was subjected to electrophoresis at pH 6.5, and radioactivity was detected by scintillation counting of 1 cm strips. A strongly arginine-positive band was observed with mobility equal to that of the *N*-terminal pentapeptide (Pettigrew *et al.*, 1975), and this contained 95% of the radioactivity on the paper. The remaining 5% was associated with the *N*-terminal tripeptide (Pettigrew *et al.*, 1975).

After acid hydrolysis and electrophoresis at

Table 3. *Specific radioactivity of products of limited tryptic digestion of Crithidia cytochrome c-557*
 Portions of each were taken for amino acid analysis and liquid-scintillation counting of radioactivity. Full experimental details are given in the Methods section.

	Specific radioactivity (c.p.m./nmol)
Cytochrome <i>c</i> -557 (before digestion)	1020
Cytochrome <i>c</i> -557 (after digestion)	1175
Cytochrome <i>c</i> -557 (–5, 102)	6
<i>N</i> -Terminal pentapeptide	1291

pH 2.0, radioactivity was associated with the unresolved band containing trimethyl-lysine and arginine, and the dimethylproline band.

After acid hydrolysis, treatment with dansyl chloride and chromatography of the Dns-amino acids on sheets of polyamide, radioactivity was associated with the Dns-trimethyl-lysine spot (which also contained dimethylproline as an unresolved undansylated component in the solvent system used). No radioactivity was found in the Dns-arginine, Dns-proline or Dns-alanine.

Quantitative amino acid analysis of the *N*-terminal pentapeptide gave Pro_(1.03), Ala_(1.04), Me₃Lys_(0.89), Arg_(1.07) (maximum impurity Gly_(0.13)). In all 48 nmol was recovered, and this is consistent with a typical 50% recovery of peptide from paper and a nearly complete digestion at the Arg–6–Glu–5 bond.

Specific radioactivity of the products of limited tryptic digestion of Crithidia cytochrome c-557

The specific radioactivities of the undigested *Crithidia* cytochrome *c*-557, the cytochrome *c* (–5, 102) and the *N*-terminal pentapeptide are compared in Table 3 with that of the cytochrome *c*-557 used for the digestion. These results demonstrate that essentially all the [¹⁴C]methyl-group incorporation occurred at the *N*-terminal region of the molecule at proline–10 and lysine–8.

Discussion

Methionine as a source of methyl groups for the methylation of Crithidia cytochrome c-557

The results in Table 1 show that the methyl group of methionine is the sole donor for methyl transfer to trimethyl-lysine and dimethylproline, presumably via *S*-adenosylmethionine. This is supported by the studies performed *in vitro* with partially purified methyltransferase.

In contrast, Lederer *et al.* (1977) have proposed that the methyl groups in *Escherichia coli* ribosomal protein L11 may come from two sources, one being independent of *S*-adenosylmethionine.

Cytochrome c-557 as a substrate for methyl transfer

In spite of the fact that *Crithidia* cytochrome *c*-557 was known to contain 2 mol of trimethyl-lysine and 1 mol of dimethylproline/mol, the cytochrome was a much better methyl acceptor than other cytochromes *c* tested, some of which contained no methylated lysine residues. Amino acid analysis of *Crithidia* cytochrome *c*-557 showed the presence of small amounts of monomethyl- and dimethyl-lysine (Fig. 1), and, if the radioactive uptake is calculated relative to this small percentage of 'unfilled' methylation sites, the reaction with cytochrome *c*-557 proceeds much farther to completion than that observed for horse cytochrome *c* with *Neurospora* cytochrome *c*-specific methyltransferase (Polastro *et al.*, 1978; Table 2).

The small amount of substrate methylation (approx. 0.2%) that was obtained for the *Neurospora* and yeast methyltransferases has been a worrying and unresolved feature of these studies. Several explanations have been put forward to account for this, which argue that the native cytochrome is not the true substrate and that physiological methylation occurs on the nascent polypeptide chain or the apocytochrome, but these are not entirely convincing. We propose that the activity reported in the present paper for *Crithidia* methyltransferase is less susceptible to this criticism.

A major problem during the present work has been the procurement of a cytochrome *c*-557 preparation that is highly active as a methyl acceptor. Different preparations of the cytochrome give very different results in the standard assay, and their effectiveness in accepting methyl groups from *S*-adenosylmethionine is closely correlated with the proportion of monomethyl- and dimethyl-lysine residues observed on amino acid analysis. However, attempts to obtain less fully methylated cytochrome, by, for example, growing cells in ethionine or harvesting cells early in growth, have been unsuccessful.

Cytochrome c methyltransferase activity of Crithidia

By incorporating an adsorption step on CM-

cellulose in the assay procedure developed by Nochumson *et al.* (1977), we were able to eliminate almost all the endogenous incorporation of label that occurs in the crude extracts in the absence of added cytochrome. This is in contrast with the studies with the *Neurospora* and yeast systems, where the incorporation of label measured after trichloroacetic acid precipitation in the absence of added cytochrome can be high in crude extracts (DiMaria *et al.*, 1979).

Material consistent with the presence of [$Me-^{14}C$]dimethylproline and [$Me-^{14}C$]trimethyl-lysine was present after paper electrophoresis at pH 2.0 of a hydrolysate of enzymically methylated cytochrome *c*-557, and all the radioactivity in the intact cytochrome was lost by removal of the *N*-terminal pentapeptide by mild trypsin treatment. The radioactivity in this peptide was contained in trimethyl-lysine (95%) and in dimethylproline (5%) residues.

Thus only the lysine residue at position -8 and the *N*-terminal proline residue are capable of accepting methyl groups in the assay system used, even though both positions are already almost fully methylated. The evidence for this is as follows. If we assume that lysine-72 is fully methylated (Pettigrew *et al.*, 1975), we can assess the degree of methylation of lysine-8 by amino acid analysis. This procedure cannot be applied to the dimethylproline, but the absence of detectable *N*-terminal proline and the stoichiometry of methyl groups deduced from the n.m.r. analysis of the *N*-terminal peptide (Smith & Pettigrew, 1980) suggest that methylation of the α -amino group is almost complete.

We cannot determine whether lysine-72 might also act as a methyl acceptor for this enzyme activity, because this residue is fully methylated in the isolated cytochrome. However, the poor reactivity of horse cytochrome *c*, which contains unmethylated lysine-72, suggests that the *Crithidia* methyltransferase is not able to methylate this lysine residue in the native cytochrome.

Other proteins tested, such as ovalbumin and histone, gave degrees of incorporation similar to the low values detected with horse cytochrome *c*.

Two preliminary lines of evidence suggest that different methyltransferases exist for the methylation of proline and lysine-8 residues in *Crithidia*. Firstly, different ratios of radioactivity in trimethyl-lysine and dimethylproline were obtained when the concentration of *S*-adenosylmethionine was varied. Secondly, different fractions obtained from DEAE-cellulose chromatography of the extract produced different ratios of radioactivity in the two amino acids in the standard assay conditions.

The function of cytochrome methylation is still in doubt. The original observation by Scott & Mitchell (1969) was that in *Neurospora* only the methylated cytochrome *c* could bind to the mitochondria. In

yeast, which also contains both methylated and unmethylated cytochrome *c*, the methylated form has a considerably higher affinity for the cytochrome oxidase (Polastro *et al.*, 1978). In these cases, methylation is at lysine-72, and this residue has been implicated in many studies of cytochrome *c* interaction with oxidases (reviewed by Rieder & Bosshard, 1980).

With *Crithidia* cytochrome *c*-557 it seems unlikely, on the basis of the X-ray crystallographic evidence for horse cytochrome *c* (Dickerson *et al.*, 1971), that the *N*-terminal region is close to the binding surface for the oxidase. An alternative possibility is that the extensive *N*-terminal methylation is a form of protection against proteolytic attack, as has been shown *in vivo* for yeast cytochrome *c* methylated lysine-72 (Farooqui *et al.*, 1981).

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