Enzymatic Methylation of in Vitro Synthesized Apocytochrome c Enhances Its Transport into Mitochondria*

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The gene for iso-1-cytochrome c from Saccharomyces cerevisiae was recloned into a pSP65 vector containing an active bacteriophage SP promoter. The iso-1-cytochrome c gene was cloned as an 856-base pair XhoI-HindIII fragment. When the resulting plasmid was digested at the HindIII site 279 bases downstream from the termination codon of the gene and transcribed in vitro using SP6 RNA polymerase, full length transcripts were produced. The SP6 iso-1-cytochrome c mRNA was translated using a rabbit reticulocyte lysate system, and the protein products were analyzed on sodium dodecyl sulfate-polyacrylamide gels. One major band with a molecular weight of 12,000 was detected by autofluorography and coincided with the Coomassie staining band of apocytochrome c from S. cerevisiae. The product was also shown to be identical with that of standard yeast apocytochrome c on an isoelectrofocusing gel. The in vitro synthesized iso-1-apocytochrome c was enzymatically methylated by adding partially purified S-adenosyl-L-methionine:cytochrome c-lysine N-methyltransferase (protein methylase III, EC 2.1.1.59) from S. cerevisiae along with S-adenosyl-L-methionine to the in vitro translation mixtures. The methylation was shown to be inhibited by the addition of the methylase inhibitor S-adenosyl-L-homocysteine or the protein synthesis inhibitor puromycin. The principal type of methylated amino acid in the protein was found to be ε-N-trimethyllysine which accounted for 77% of the total. Finally, the methylation of in vitro synthesized iso-1-apocytochrome c was found to increase its import into mitochondria isolated from S. cerevisiae 2–4-fold over unmethylated protein, but not into rat liver mitochondria. This suggests that methylation facilitates the import of apocytochrome c into mitochondria by a specific receptor mechanism.

Protein methylation is one of the most ubiquitously occurring post-translational side-chain modification reactions of protein (1–5). Its precise biochemical function is well established in only a few systems: in carnitine biosynthesis by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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**Materials—SP6 RNA polymerase was obtained from Boehringer Mannheim. Guanidyltransferase was purchased from Bethesda Research Laboratories. Rabbit reticulocyte lysate (nuclease treated), pSP65 plasmid, and RNasin were obtained from Promega Biotec, Madison, WI. rNTPs were obtained from Pharmacia Biotechnology, Inc. AdoMet (iodine salt), S-adenosyl-L-homocysteine (AdoHcy), puromycin, ampicillin, and cytochromes c from yeast and horse heart were purchased from Sigma. [3H]METHYLMETHIONINE (500 Ci/mmol) was obtained from ICN, Irvine, CA. L-[3H]Leucine (70 Ci/mmol) and S-adenosyl-L-[methyl-3H]methionine (85 Ci/mmol) were purchased from Amer.

EXPERIMENTAL PROCEDURES

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In Vitro Synthesis and Methylation of Apocytochrome c

Sham Corp. All other chemicals were obtained from various commercial sources and were of the highest purity grade available. Apocytochrome c was prepared from yeast holocytochrome c by removal of the heme using AgSO\(_4\) (20).

**Purification of AdoMet:Cytochrome c-Lysine N-Methyltransferase** (Protein Methylyase III) from Yeast—Protein methylase III was purified as described (14) with the following exceptions. The calcium phosphate gel step was omitted from the procedure. The DEAE-cellulose column was eluted with a linear 0-0.4 M KCl gradient in the buffer described. After DEAE-column chromatography the enzyme pool was concentrated to 6 ml using a CX-10 filter, divided into aliquots, and frozen at -70 °C. The Sephadex column was necessary in order to rid the enzyme preparation of a small molecular weight compound that inhibited the translation reaction.

**Isolation of Mitochondria**—Mitochondria were prepared from *S. cerevisiae* according to the method of Mattson and Balacavage (21) except that cells were broken in a French pressure cell at 8,000-10,000 p.s.i. and Cytochrome c depletion of the mitochondria was performed according to the method of Jacobs and Sanadi (22) as modified by Boveris et al. (23). Rat liver mitochondria were prepared from freshly killed rats according to the method of Williams and Thorp (24).

**Plasmid Construction**—The plasmid pSP65-CYC1(0.8) was constructed by cloning an 856-bp Xhol-HindIII fragment from plasmid YEp13CYC1(2.5) into SalI-HindIII-digested pSP65 (25, 26). The plasmid was then transformed into *E. coli* strain BW2019 and the plasmids were isolated from bacteria. The plasmids were then purified by the method of Sambrook et al. (27).

**In Vitro Transcription with SP6 RNA Polymerase**—Twenty-five μg of plasmid pSP65-CYC1(0.8) was linearized in a 50-μl reaction mixture of HindIII digestion buffer (Boehringer Mannheim, England Biosabs) for 1.5 h at 37 °C (27). The reaction mixture was then extracted with phenol/chloroform (1:1). The linearized plasmid was precipitated and centrifuged. The pellet was washed with ethanol, and the dried pellet was dissolved in 25 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

**Addition of the 7-Methylguanine Cap (MeGTP)**—MeGTP was added to SP6 mRNAs (2.0-3.0 μg of mRNA was processed for each reaction) using guanylyltransferase in a 50-μl reaction mixture as described (27). Capping was shown to increase translational activity as much as 3-5 fold (data not shown).

**Cell-free Translation**—The mRNA pellet after the capping was dissolved in 18 μl of diethylpyrocarbonate-treated water and either used for translation or stored at -20 °C. The *in vitro* translation mixture in which synthesis of iso-1-apocytochrome c was being studied contained 5 μCi of [\(^3H\)leucine, 10 μl of rabbit reticulocyte lysate, 0.3-μl amino acid mixture (1 mM each amino acid, minus leucine), 8 μl of diethylpyrocarbonate-treated water, 0.1 μl of RNasin (3.6 units), and 0.2 μg of iso-1-cytochrome c mRNA. Routinely, a reaction mixture for the assays was prepared by adding rabbit reticulocyte lysate, amino acid mixture, water, and RNasin to 50 μCi of [\(^3H\)leucine. This mixture was then equally divided into 10 assay tubes (3-μl aliquots). Each reaction (13 μl) was preincubated at 30 °C for 5 min, mRNA was then added to the assay tubes (final volume of the assay mixture was 18.5 μl), and the mixtures were incubated at 37 °C for 60 min. At the end of the incubation period, 10 μl of the reaction mixture was added to 6.5 ml of 1 M NaOH, 1.5% H\(_2\)O\(_2\). This mixture was heated at 37 °C for 10 min. Two milliliters of 25% trichloroacetic acid containing 2% casein hydrolysate was then added to the mixtures. They were cooled for at least 30 min in an ice bath. The mixtures were filtered through glass fiber filters and washed successively with 6 ml each of 8% trichloroacetic acid and 20% ethanol. The filters were then dried under an infrared lamp for 5 min. The dried filters were placed in counting vials along with 5 ml of scintillant (ScintiVerse, Fisher) and counted for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer with a disintegrations/min converter.

**Methylation of in Vitro Synthesized Apocytochrome c by Protein Methylyase III**—Translation mixtures in which methyl group incorporation was being studied contained 10 μl of rabbit reticulocyte lysate, 0.3 μl of amino acid mixture, 4 μl of diethylpyrocarbonate-treated water, 0.1 μl of RNasin (3.6 units), 0.2 μg of iso-1-cytochrome c mRNA, 1.75 μl of partially purified protein methylase III (2.2 μg of protein), and 2.5 μl of Ado(methyl-\(^3H\)Met (2.5 Ci/ml). As above, a mixture was prepared and added to assay tubes containing protein methylase III, Ado(methyl-\(^3H\)Met, and mRNA. Assays were then processed for activity as described above.

**Effect of in Vitro Synthesized Apocytochrome c Methylation on Its Import into Mitochondria**—For measurement of iso-1-apocytochrome c import to the mitochondria, the *in vitro* translation system described above was employed. Methylation of the newly synthesized protein was also performed as described; however, unlabeled AdoMet at a final concentration of 0.1 mM was used in place of labeled AdoMet (instead, \[^3H\]leucine was used). For methylation inhibition studies, the same concentration of AdoMet and protein methylase III were used, and AdoHcy at a final concentration of 0.2 mM was added.

Translation and methylation (where appropriate) were allowed to proceed for 1 h at which time 40 μl of the mitochondrial preparation (approximately 200-400 μg of protein) were added. The reaction mixtures were incubated for 1 h at 30 °C. After this incubation, 150 μl of the mannnitol-sucrose-MOPS buffer, pH 7.2, (mitochondrial preparation buffer) were added, and the mixtures were centrifuged in an Eppendorf microcentrifuge for 10 min. The supernatants were removed, and the pellets were washed twice with 150 μl of the same buffer.

After washing, the pellets were suspended in 50 μl of the mannnitol-sucrose-MOPS buffer and were treated with trypsin at a final concentration of 0.1 μg/μl (29). This treatment removes any nonspecifically bound radioactive protein(s). Trypsin treatment took place at 0 °C for 30 min after which the mixtures were centrifuged and washed twice more before these pellets were then treated with 30 μl of 3 M NaOH, 1.5% H\(_2\)O\(_2\) at 37 °C for 5-10 min until no color was noticeable and were counted for radioactivity.

**Ge! Electrophoresis**—Electrophoresis of RNA was performed on 1.5% agarose gels as described (28). Proteins were analyzed by electrophoresis in SDS-polyacrylamide gels (30) containing 15% acrylamide. SDS-polyacrylamide gels were stained with 0.1% Coomassie Brilliant Blue containing 30% methanol and 7% acetic acid. After destaining in 30% methanol, 7% acetic acid, the gels were treated for 1 h with ENHANCE (Du Pont-New England Nuclear), dried, and autoradiographed using Kodak XAR-5-Omat film.

**Isoelectrofocusing**—Isoelectrofocusing gels were prepared as described by O'Farrell (31) with the following changes. For the preparation of 10 gels the pH gradient was made by mixing 1.25 ml of Ampholine (pH range 9-11) and 0.21 ml of Ampholine (pH range 3.5-10). The gels were prerun as described; however, when the samples were loaded the gels were focused at 400 V for 6 h. After focusing was complete, the gels were cut into 5-mm sections. The pH gradient was determined by soaking the gel slices in 2 ml of degassed water for at least 4 h and then reading the pH of each slice using a Radiometer pHMS82 pH meter. Radiolabeled gels were cut into 5-mm sections, and the sections were placed in counting vials with 5 ml of scintillation solution and counted for radioactivity.

**Analysis of Methylated Amino Acids**—Proteins were hydrolyzed for 18 h in 6 N HCl at 110 °C in vacuo. The acid was removed by evaporation, and the residue was washed four times with deionized water followed by evaporation. The hydrolysates were then analyzed on an automatic amino acid analyzer. Radioactivity was determined by collecting fractions from the column and counting an aliquot from each fraction in a scintillation spectrometer. More detail of this procedure is given in the legend for Fig. 5.
RESULTS

Recloning and Transcription of the Iso-1-cytochrome c Gene in a SP6 Promoter System—The gene for iso-1-cytochrome c of yeast was cloned as an 866-bp XhoI-HindIII fragment into the vector pSP65 to form pSP65-CYC1(0.8). This plasmid contains the entire iso-1-cytochrome c gene from yeast as well as 250 bp upstream from the AUG start codon (Fig. 1). Taking into account the polylinker region of the pSP65 vector, the entire leader sequence transcribed 5' to the start codon of the gene is 286 bp. pSP65-CYC1(0.8) contains one HindIII site 279 bp downstream from the stop codon of the iso-1-cytochrome c gene. When linearized at this HindIII site and utilized as a template in the SP6 RNA polymerase assay, full-length runoff transcripts were produced (top panel of Fig. 1). Translation of the SP6-transcribed mRNA—The synthetic iso-1-cytochrome c mRNA after capping with 7-mGpppNp was utilized as a substrate in the rabbit reticulocyte lysate assay. Incorporation of L-[3H]leucine into the trichloroacetic acid-insoluble material increased linearly with the incubation time up to 15 min after which time the activity began to level off (data not shown). As little as 0.01 μg of mRNA was needed in order to obtain measurable activity with the in vitro translation assay, a linear increase in L-[3H]leucine incorporation up to 0.05 μg of mRNA. Further increases in the amount of mRNA caused only a gradual increase in L-[3H]leucine incorporation up to 0.2 μg which was the concentration that gave the maximum activity (data not shown).

Identification of the in Vitro Translation Product as Yeast Apocytochrome c—The protein(s) synthesized in the translation mixture with synthetic iso-1-cytochrome c mRNA was characterized on SDS-polyacrylamide gels with yeast apocytochrome c as a standard marker (Fig. 2). As seen in Fig. 2, when iso-1-cytochrome c mRNA was used as a template, one single radiolabeled band migrated in the corresponding region of apocytochrome c (lane d). This band is absent in lane c, which represents endogenous activity in the rabbit reticulocyte lysate. Standard apocytochrome c from yeast is represented in lane b. The in vitro translated apocytochrome c also focused with standard apocytochrome c on isoelectrofocusing gels (Fig. 3); both the in vitro translated product and standard apocytochrome c focused at a pH of 9.48.

Methylation of the in Vitro Synthesized Apocytochrome c—Newly synthesized apocytochrome c was methylated by incubating partially purified protein methy lase III from yeast along with Ado[methyl-3H]Met in the in vitro translation assay. Apocytochrome c was identified as the methylated product using SDS-polyacrylamide gel electrophoresis. As seen in Fig. 2 (lanes e and f), the major radiolabeled band formed when cytochrome c mRNA was incubated in the presence of protein methylase III and Ado[methyl-3H]Met corresponding to standard apocytochrome c from yeast was synthesized in the in vitro translation assay and electrophoresed on a 1.5% agarose gel. The gel was then dried and autoradiographed.

FIG. 1. In vitro transcription of SP6 iso-1-cytochrome c mRNA molecules. The diagram shown at the bottom of the figure represents the DNA template used in synthesizing iso-1-cytochrome c mRNA molecules with SP6 RNA polymerase. The top panel of the figure shows an autoradiograph of the RNA species made in the transcription assay and electrophoresed on a 1.5% agarose gel. The RNA molecules were labeled in the transcription assay by adding 50 μCi of [γ-32P]GTP to the reaction mixture as described under "Experimental Procedures." The gel was then dried and autoradiographed. Lane A represents transcribed products of a positive control template (Promega). Sizes of each RNA species are indicated. Lane B represents mRNA formed from pSP65-CYC1(0.8), linearized with HindIII (886 bases).

FIG. 2. SDS-polyacrylamide gel electrophoresis of in vitro translated and methylated products. Two-tenth μg of SP6 iso-1-cytochrome c mRNA were incubated in the in vitro translation assay for 1 h as described. Five μl of the reaction mixtures were then loaded onto the gel. Lanes a and b represent Coomassie-stained proteins. Lane a, standard molecular weight marker proteins: bovine albumin (M, 66,000), egg albumin (M, 45,000), glyceraldehyde-3-phosphate dehydrogenase (M, 36,000), carbonic anhydrase (M, 29,000), trypsinogen (M, 24,000), trypsin inhibitor (M, 20,100), α-lactalbumin (M, 14,200); lane b, standard yeast apocytochrome c. Lanes c and d represent autoradiographs of the in vitro translation products labeled with L-[3H]leucine. Lane c, translation mixture with no added mRNA (endogenous control); lane d, translation mixture with SP6 iso-1-cytochrome c mRNA. For the analysis of methylation (lanes e and f), SP6 iso-1-cytochrome c mRNA (0.2 μg) was translated in the presence of cytochrome c-lysine N-methyltransferase (protein methylase III) and Ado[methyl-3H]Met as described under "Experimental Procedures." Eight μl of the reaction mixtures were then loaded onto the gel. Lanes e and f represent autoradiographs of the methylated in vitro translation products. Lane e, translation mixture with no added mRNA (endogenous control); lane f, translation mixture with SP6 iso-1-cytochrome c mRNA and methylated. The large arrow indicates the migration of apocytochrome c.
yeast (lane b). This band was absent in lane c which represents endogenous methylation in the reticulocyte lysate when no iso-1-cytochrome c mRNA is added.

In order to identify the methylated amino acids formed in the translation assay, the methyl-\(^{3}H\)-labeled proteins were hydrolyzed with acid and amino acids chromatographed using an automatic amino acid analyzer. Three radiolabeled peaks were found when iso-1-cytochrome c mRNA was incubated with the lysate and protein methylase III (Fig. 4). These peaks are absent in the assay mixture in which cytochrome c mRNA was omitted. When compared with standard methylated amino acids, these derivatives cochromatographed with \(\epsilon-N\)-mono-, \(\epsilon-N\)-di-, and trimethyllysine. \(\epsilon-N\)-Trimethyllysine was present in the greatest amount (77%) whereas \(\epsilon-N\)-monomethyl- and dimethyllysine were present in smaller concentrations, 9 and 14%, respectively.

**Effect of Various Inhibitors on Apocytochrome c Synthesis and Methylation**—The methylation of *in vitro* translated apocytochrome c was found to be inhibited by both AdoHcy and puromycin. AdoHcy is the product formed from AdoMet after methyl group transfer and is an excellent competitive inhibitor of all known AdoMet-dependent methyltransferases. Inclusion of 0.2 mM AdoHcy in the translation mixtures along with cytochrome c mRNA inhibited methylation by 94% (Table I) while having little effect on cytochrome c synthesis. This data is additional evidence indicating that methylation of *in vitro* synthesized apocytochrome c is AdoMet-dependent.

Puromycin, an antibiotic inhibitor of protein biosynthesis, inhibited methylation by 81% at a concentration of 1.0 mM. This same concentration of puromycin inhibited synthesis by 98%. It must be noted, however, that the methylation level obtained when puromycin was included in the assay is below that of the endogenous level where no mRNA was added. This suggests that methylation occurring in the presence of puromycin is not that of cytochrome c (see also lane e of Fig. 2). Use of puromycin also demonstrates that synthesis of apocytochrome c is a requirement for lysine methylation of this protein.

**Effect of Methylation of *in Vitro* Synthesized Apocytochrome c on Its Import to Mitochondria**—As Table II clearly shows, methylation of newly synthesized apocytochrome c enhances its import to isolated yeast mitochondria 2–4-fold. In general, more apocytochrome c, both methylated and unmethylated, was taken up by cytochrome c-depleted mitochondria. However, the increase due to methylation was observed in both cytochrome c-depleted and undepleted mitochondria, more prominently with the former. In contrast to the above, the methylation did not influence the import of *in vitro* synthesized apocytochrome c into rat liver mitochondria.

It should be noted in the table that when all the necessary components of methylation were present in the assay but methylation was inhibited by AdoHcy, no increase in uptake by the yeast mitochondria was observed. The apparent decrease in uptake shown when both AdoMet and AdoHcy were present in the assay (lines 2 and 5) is within the range of experimental error.

**DISCUSSION**

The major goal of this project was to develop a cytochrome c methylation system most like that which occurs *in vivo* and to utilize it for investigating some of the possible biochemical...
functions of enzymatic methylation in a cell-free environment. Thus, the gene for iso-1-cytochrome c from *S. cerevisiae* was cloned into a pSP65 vector containing an active bacteriophage SP6 promoter, and mRNA for iso-1-apocytochrome c was synthesized. This system proved to be much more efficient and much less time-consuming than isolating cytochrome c mRNA by the selective hybridization technique described by Boss *et al.* (32). In the selective hybridization procedure, poly(A)* mRNA was isolated from 70–85 g of yeast, cytochrome c mRNA could be produced from chosen for these studies because it contained lower endogenous protein methylase activity. The attachment of a methyl group to cytochrome c was identified as lysine by amino acid analysis. The extent of lysine derivatives are derived from apocytochrome c.

The data from the SDS-polyacrylamide gel and the isoelectrofocusing gel show that only one protein product is produced during the translation of iso-l-cytochrome c mRNA. The labeled product was found to migrate in the same region as apocytochrome c from *S. cerevisiae* in both gel systems (Figs. 2 and 3). Both standard apocytochrome c from yeast and the labeled translation product focused at a pl value of 9.48 (Fig. 3). Considering these two methods of identification, we feel very confident that the only in vitro translation product using iso-l-cytochrome c mRNA as a template is iso-l-apocytochrome c. This also confirms an earlier observation that apocytochrome c synthesized in vitro does not contain an additional signal peptide (33).

Newly synthesized iso-l-apocytochrome c was methylated in vitro by adding cytochrome c-lysine *N*-methyltransferase (protein methylase III) and *Ado[methyl-^3^H]Met* to the reaction mixture (Fig. 2). Incorporation of methyl groups was shown to require the presence of newly synthesized iso-l-apocytochrome c in the reaction mixture (Table I) and was inhibited by the addition of *AdoHcy*, which is a potent competitive inhibitor for *AdoMet*. The methylated amino acid in apocytochrome c was identified as lysine by amino acid analyzer (Fig. 4). L-^N^-Mono-lysine was present only in the acid hydrolysates of translated protein in which cytochrome c mRNA was incubated. These amino acids were absent in the endogenous assay suggesting that all methylated lysine derivatives are derived from apocytochrome c.

We have earlier observed that only 0.2 mol % of "native" horse heart cytochrome c could maximally be methylated after prolonged incubation with an excess amount of *Neurospora* cytochrome c-lysine *N*-methyltransferase (13). The extent of
methylation could be increased to 3.6% with ethanol-denatured cytochrome c. This suggested to us that the extent of enzymatic methylation is greatly dependent on the conformational state of the substrate protein. In confirming the above suggestion, the results presented in Table I clearly demonstrate that at least 17% of newly in vitro synthesized apocytochrome c can be trimethylated by the simultaneous presence of cytochrome c-lysine N-methyltransferase. This calculation is based on the following considerations. Under present experimental conditions, 1.37 pmol of [3H]leucine is incorporated into apocytochrome c (6th column), and since 1 mol of yeast apocytochrome c contains 8 mol of leucine, this corresponds to 0.17 pmol of apocytochrome c synthesized. On the other hand, 0.11 pmol of methyl-[3H] is incorporated into the protein (3rd column) and 77% of the methyl-[3H] incorporated is found in the form of trimethyllysine (Fig. 4). This is equivalent to 0.028 pmol of trimethyllysine. Therefore, 17% (0.028/0.170 × 100) of the newly in vitro synthesized apocytochrome c has been trimethylated.

The most striking observation of this paper involves the difference in uptake between methylated and unmethylated apocytochrome c in yeast and rat liver mitochondria. Enzymatic methylation of newly synthesized iso-1-apocytochrome c was found to increase its import into yeast mitochondria (Table II), but not into rat liver mitochondria. It is conceivable that methylation confers increased resistance to intracellular proteolytic degradation rather than increasing specific import into the mitochondria (by maintaining a higher concentration of apocytochrome c); however, this does not appear to be the case. First, Van Noort et al. (34) observed that methylation of lysine-65 of elongation factor Tu from Escherichia coli does not increase resistance to tryptic degradation. More specifically, Pola, et al. (18) showed that methylated and unmethylated iso-1-cytochrome c isolated from yeast were digested at equal rates by trypsin and yeast protease A and B. These two findings argue against resistance to proteolytic degradation due to methylation. Within the scope of this paper, these previous observations support the contention that methylation increases the import of apocytochrome c into yeast mitochondria rather than decreasing its susceptibility to protease action.

Another more plausible explanation involves a specific receptor which recognizes the methylated apocytochrome c more readily than the unmethylated protein. Although no direct evidence is available to involve any particular mitochondrial component, the existence of a receptor for apocytochrome c in mitochondria of eukaryotic organisms has been well documented (35). Matsuura, et al. (36) have examined the import of radiolabeled rat apocytochrome c into rat liver mitochondria and observed that a 3000-fold excess of unlabeled chemically prepared apocytochrome c but not holocytochrome c from either rabbit or horse could inhibit this import. There is also evidence that the ligand specificity for binding apocytochrome c to Neurospora mitochondria involves species specificity; equine apocytochrome c was an order of magnitude less effective in displacing bound Neurospora apocytochrome c than the homospecific protein, and a bacterial apocytochrome c was not effective (37). These observations argue in favor of receptor-mediated uptake of apocytochrome c by mitochondria.

In line with these reports, we observed in this paper that rat liver mitochondria makes no distinction between the two protein species, methylated or unmethylated, while yeast mitochondria does. While providing evidence that yeast mitochondria also possess a receptor for apocytochrome c, this suggests that this receptor prefers methylated apocytochrome c for uptake into the mitochondria. The mechanism involved in this preference is not clear at present. It is possible that the receptor might recognize the change in pl value caused by methylation of apocytochrome c at lysine-72 (38). The decrease in pl of methylated protein is the most noticeable difference yet observed between the two protein species and is the most obvious choice for distinction between the two forms.

Alternative to (or perhaps in conjunction with) the receptor having a preference for methylated apocytochrome c, it is possible that the cytochrome c heme lyase (heme-attaching enzyme) (39) may prefer the methylated species. The heme lyase might attach the heme group preferentially (or exclusively) to methylated apocytochrome c, increasing the flux of methylated apocytochrome c into the mitochondria. It has been observed that inhibition of heme attachment to apocytochrome c in Neurospora mitochondria also inhibits uptake by the mitochondria and that reversal of the inhibition allows complete translocation of the protein into the mitochondria (40). Furthermore, only methylated cytochrome c was extractable from the poky mutant of Neurospora (41). Combination of these observations implicates the possible importance of methylation of apocytochrome c in the import into the mitochondria and/or in the attachment of the heme group to form holocytochrome c.

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In Vitro Synthesis and Methylation of Apocytochrome c