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Cytochrome *C* methylation: Current Knowledge of its Biological Significance

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Abstract□The yeast cytochrome *c* gene has been recloned, and the resulting cytochrome *c* mRNA has been translated in rabbit reticulocyte lysate translation system. The newly synthesized apocytochrome *c* could be methylated by exogenously added cytochrome *c*-lysine N-methyltransferase. Enzymatic methylation of *in vitro* synthesized apocytochrome *c* was found to facilitate specifically its import into mitochondria of yeast, but not of rat liver.

Key words□Protein methylation, Cytochrome *c* methylation.

INTRODUCTION

Protein Methylation occurs ubiquitously in nature, in organisms ranging from prokaryotic to eukaryotic. The reaction involves N-methylation of lysine, arginine, histidine, alanine, proline and glutamine, O-methylation of glutamic and aspartic acid, and S-methylation of cysteine and methionine[1-3].

In nature, methylated amino acids occur in highly specialized proteins such as histones, flagella proteins, myosin, actin, ribosomal proteins, hnRNA-bound protein, HMG-1 and HMG-2 protein, fungal and plant cytochrome *c*, myelin basic protein (MBP), opsin, EF-Tu, EF-Iα, porcine heart citrate synthase, calmodulin, ferredoxin, α-amylase, heat shock proteins, scleroderma antigen, nucleolar protein C23, and IF-3[4].

During the past two decades, this laboratory has revealed that these methylations are carried out by several classes of highly protein-specific methyltransferases[5-7]. For example, protein methylase I (S-adenosyl-L-methionine: protein-arginine N-methyltransferase; EC 2.1.1.23), representing one such class, methylates the guanidino group of arginine residues; protein methylase II (S-adenosyl-L-methionine: protein-carboxyl O-methyltransferase; EC 2.1.1.24) methylates the carboxyl group of glutamyl or aspartyl residues; and protein methylase III (S-adenosyl-L-methionine: protein-lysine N-methyltransferase; EC 2.1.1.43) methylates the ε-amino group of lysine residues[3].

The most remarkable characteristic of these methyltransferases is the high degree of specificity toward a particular amino acid residue in the substrate protein. However, there is increasing evidence of an additional level of specificity in the identify of the methyl-acceptor protein species. At least several examples of each of these methyltransferase classes have been characterized: Histone-[7,8], cytochrome *c*-[9], and calmodulin-specific[10] protein methylase III's or histone-[11] and MBP-specific[12] protein methylase I's.

In recent years, the significance of protein methylation in biochemical and cellular processes has become increasingly evident. Protein-bound ε-N-trimethyl-L-lysine serves as a metabolic precursor for carnitine biosynthesis[13]. MCP (methyl-accepting chemotactic protein)-glutamyl methylester is a key biochemical signal for sensory input in bacterial chemotaxis[14,15]. Moreover, protein-bound cysteine residues in O^6-methylguanine-DNA methyltransferase have been observed to accept methyl groups from methylated DNA during DNA repair[16].

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CYTOCHROME C METHYLATION

During the past decade or so, our attention has focused mainly on cytochrome *c* methylation (reviewed in references 17-19). To gain a better understanding of the protein methylation reaction, we chose cytochrome *c* as a model system. Cytochrome *c* is present in essentially all eukaryotes, is easily purified, and is relatively small in size (approximately 100 amino acid residues). Not surprisingly, it has been one of the most thoroughly studied proteins. Over 80 sequences from a wide variety of eukaryotic species have been determined. Crystal structures from three species, horse, tuna, and bonito[20-22], are known from X-ray diffraction studies. Despite large sequence variations among the wide range of eukaryotic species[23], cytochrome *c* has retained a remarkable degree of consistency in biological function, tertiary structure, and a number of other important properties including redox potential[24]. This constancy of cytochrome *c* has given insight into the relationship between protein structural characteristics and primary function.

Comparison of the sequences has revealed that invariant residues occur as never more than two adjacent amino acids with one exception. This exception is the presence of an almost absolutely invariant undecapeptide extending from residue-70 to 80[23]. The conservation of this region suggests that it is of paramount importance in maintaining the integrity and/or function of the molecule. In this regard, the sequence resides in a region to the left of the heme crevice, a region where the binding of cytochrome *c* oxidase and other redox enzymes has been localized[25]. Another key observation regarding sequence homology is the invariance of the number of lysines which reside on the front face of the molecule, essentially in the area of the so-called "heme crevice", through which electron transfer is thought to occur[26]. These lysines are of extreme importance in the electrostatic binding of cytochrome *c* to the various enzymes previously mentioned, the ultimate purpose of this electrostatic binding being the proper heme alignment for efficient electron transfer[26].

It is of great and continuing importance that the residue-72 lysine in virtually all lower organisms (non-animal) is trimethylated [initially observed by DeLange *et al.*[27,28] in *Neurospora crassa* and *Saccharomyces cerevisiae*]. thus compromising the absolute invariance of the residue-70 to 80 region. Furthermore, in all plants and some protozoan organisms an additional site (residue-86) is seen to be trimethylated[27]. These methylations are more significant since both of these residues[72,86] are among the aforementioned critically important and invariant lysines.

In accordance with the observations of methylated lysine in cytochrome *c* by DeLange *et al.*, we initially identified and subsequently purified a methyltransferase from *N. crassa* which is very specific for cytochrome *c*. Of all the proteins examined, only naturally unmethylated cytochrome *c* served as a substrate[9]. It should be noted that histones were not substrates[7]. The methyltransferase was also highly residue-specific. Although there are 19 unmethylated lysines in horse heart cytochrome *c* with many on the exterior of the molecule, the enzyme methylates only a single lysine residue at position 72[29]. This position 72-lysine methylation coincided with the lysine which was methylated *in vivo* in the cytochrome *c* of yeast, wheat germ and *Neurospora*. At present, this cytochrome *c*-specific lysine N-methyltransferase (Protein methylase III; EC 2.1.1.59) has been purified from various sources; 3,500-fold from *N. crassa*[9], 63-fold from yeast[29], and 135-fold from wheat germ[30].

In characterizing these enzymes, certain important observations were made. The heme-free apocytochrome *c* served as a much better substrate for protein methylase III than holocytochrome *c*[29]. However, when bound to mitochondria in low ionic conditions, both the apo- and holo- forms of cytochrome *c* lost their substrate-capability. This capability was restored when the proteins were released from the mitochondria by KCl treatment[29]. Cycloheximide also inhibited both protein backbone synthesis and methylation. These results suggested that cytochrome *c* is methylated before heme-attachment and binding to the mitochondria at a stage concomitant with or very shortly after peptide backbone synthesis. This suggestion has been supported by pulse-chase studies showing methylation to be tightly coupled with protein synthesis[31]. The close coupling of translation and methylation has an analogue in the co-translational enzymatic methylation of *in vitro* synthesized myosin[32] and of protein directed by oviduct mRNA[33].

The biological implications of such a situation are that the methylation could, at least in principle, have a role in the elongation and/or termination of the polypeptide. Alternatively, in light of the fact that the cytochrome *c* initially enters the mitochondria in the apo- form where heme attachment subsequently occurs, methylation may facilitate transport across the outer membrane. Such a scenario is strengthened by recent findings that the transportation is receptor-mediated and the recognition site

for trans-outer membrane passage of the protein resides in the carboxyl-terminal third of the molecule[34], which of course encompasses the lysine-72 methylation site. A third possibility is that the methylation protects the protein from proteolytic attack while polysomebound or after its release.

In order to investigate the above possibilities, we have established a system in which apocytochrome *c* of yeast can be synthesized and enzymatically methylated in a totally *in vitro* cell-free system. By recloning the yeast cytochrome *c* gene into a pSP65 vector and transcribing the gene with SP6 polymerase, resulting cytochrome *c* mRNA has been translated in rabbit reticulocyte lysate translation assay[35]. The protein produced proved to be full length apocytochrome *c*. The newly synthesized apocytochrome *c* could be methylated by exogenously added cytochrome *c*-lysine N-methyltransferase (EC 2.1.1.59) and S-adenosyl-L-methionine. Enzymatic methylation of *in vitro* synthesized apocytochrome *c* was found to facilitate specifically its import into mitochondria of yeast, but not of rat liver. A summary of the results obtained is described below.

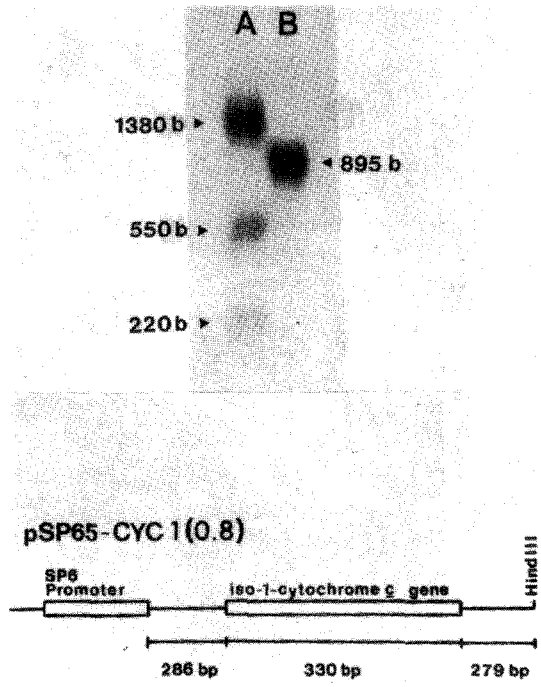

Fig. 1. In vitro transcription of SP6 iso-1-cytochrome c mRNA molecules
The diagram shows at the bottom of the figure represents the DNA template used in synthesizing iso-1-cytochrome *c* mRNA molecules with SP6 RNA polymerase. The top panel of the figure shows an autoradiograph of the RNA species made in the transcription assay and eletrophoresed on a 1.5% agarose gel. The RNA molecules were labeled in the transcription assay by adding 50 μCi of ^{32}P-α-GTP to the reaction mixtures. 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-CYCl(0.8), linearized with Hind III (895 bases).

Recloning and transcription of the iso-1-cytochrome c gene in an SP6 promoter-system

The gene for iso-1-cytochrome *c* of yeast was cloned as an 856 bp Xho I-Hind III fragment into the vector pSP65 to form pSP65-CYCl(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-CYCl(0.8) contains one Hind III site 279 bp down-stream from the stop codon of the iso-1-cytochrome *c* gene. When linearized at this Hind III site and utilized as a template in the SP6 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 $^{7}mG_{PPP}N_{P}$ was utilized as a substrate in the rabbit reticulocyte lysate assay. Incorporation of L-(^{3}H)leucine into trichloroacetic acid-insoluble material increased linearly with the incubation time up to 15 minutes after which time the activity began to level off. 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-(^{3}H)-leucine incorporation up 0.05 μg of mRNA was observed. Further increase in the amount of mRNA caused only a gradual increase in L-(^{3}H)leucine incorporation up to 0.2 μg which was the concentration that gave the maximum activity.

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 (lanes c and d of Fig. 2). As seen in Fig. 2, when iso-1-cytochrome *c* mRNA was used as a template, one

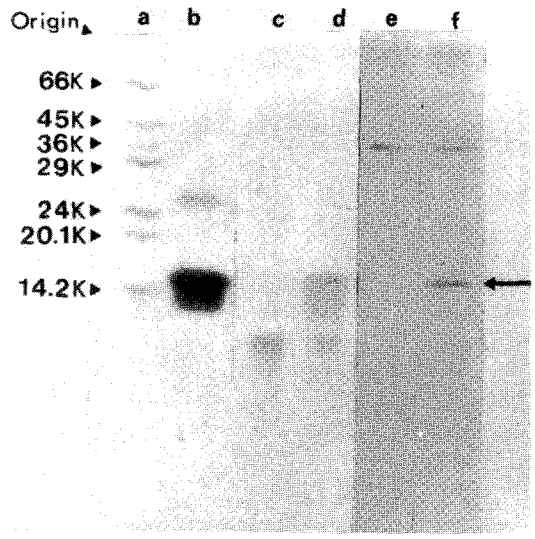

Fig. 2. SDS-polyacrylamide gel electrophoresis of *in vitro* translated and methylated products
Two-tenth μg of SP6 iso-1-cytochrome *c* mRNA were incubated in the *in vitro* translation assay for 1 hr (35). Five μl of the reaction mixtures were then loaded onto the gel. Lanes a and b represent Coomassie stained proteins. Lane a, standard molecular weight marker proteins; lane b, standard yeast apocytochrome *c*; lane c and d represent autoradiographs of the *in vitro* translation products. 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 S-adenosyl-L-(*methyl*-^{3}H)methionine. 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. Large arrow indicates the migration of apocytochrome *c*.

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 in the figure. The *in vitro* translated apocytochrome *c* also focused with that of standard apocytochrome *c* on isoelectrofocusing gels; both the *in vitro* translated product and standard apocytochrome *c* focused at a pH of 9.48

Methylation of in vitro synthesized apocytochrome c

Newly synthesized apocytochrome *c* was methylated by incubating partially purified protein methylase III from yeast along with S-adenosyl-L-(*methyl*-^{3}H)methionine in the *in vitro* translation assay.

Apocytochrome *c* was identified as the methylated product using SDS-polyacrylamide gel. As seen in lanes e and f of Fig. 2, the major radiolabeled band formed when cytochrome *c* mRNA was incubated in the presence of protein methylase III and S-adenosyl-L-(*methyl*-^{3}H)methionine (lane f) corresponding to standard apocytochrome *c* from yeast (lane b). This band was absent in lane e 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 6 N HCl and amino acids chromatographed using an automatic amino acid analyzer[9]. Three radiolabeled peaks were found when iso-1-cytochrome *c* mRNA was incubated with the lysate and protein methylase III. When compared with standard methylated amino acids, these radiolabeled derivatives co-chromatographed with ε-N-mono-, di and trimethyllysine. ε-N-Trimethyllysine was present in the greatest amount (77%) whereas ε-N-mono and dimethyllysine were present in smaller concentrations, 9 and 14%, respectively.

Effect of methylation of in vitro synthesized apocytochrome c on its import to mitochondria

As Table I clearly shows, methylation of newly synthesized apocytochrome *c* enhances its import into isolated mitochondria 2 to 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 noticeably in the former. In contrast to the above, however, methylation did not influence the import of *in vitro* synthesized apocytochrome *c* into rat liver mitochondria.

It should be noted in Table I that when all the necessary components of methylation were present in the assay but methylation was inhibited by S-adenosyl-L-homocysteine (AdoHcy), no increase in uptake by the yeast mitochondria was observed, confirming further that increase of uptake observed was due to methylation of the hemoprotein.

CONCLUDING REMARKS

Enzymatic methylation of newly synthesized iso-1-apocytochrome *c* was found to increase its import into yeast mitochondria, 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. However, this does not appear to be the case. First, Van Noort *et al.*[36] observed that methylation of lysine-56 of EF-Tu from *Escherichia coli* does not increase resistance to tryptic degradation. More specifically, Polastro *et al.*[37] showed that methylated and unmethylated iso-1-cytochrome *c* isolated from yeast were digested at equal rates by trypsin and yeast proteases A and B. These 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.

An 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[38]. Matsuura *et al.*[34] have examined the import of radiolabeled rat apocytochrome *c* into rat liver mitochondria and observed that a 3,000-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[39]. 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.

Table I. Effect of methylation of *in vitro* synthesized apocytochrome *c* on its import into mitochrondria isolated from yeast and rat liver

Source of mitochondria and treatment	Methylation		Amount imported into mitochondria		
	AdoMet*	AdoHcy	Amount of leucine (pmol)**	Leucine/mg mitochondrial protein (pmol/mg)	%
Yeast;					
depleted***	−	+	0.20	0.85	100
	+	+	0.15	0.64	75
	+	−	0.46	1.95	230
Yeast; not					
depleted	−	+	0.08	0.18	100
	+	+	0.07	0.16	88
	+	−	0.30	0.69	375
Rat liver; not					
depleted	+	+	0.03	0.09	100
	+	−	0.04	0.12	133

* The final concentrations of AdoMet (S-adenosyl-L-methionine) and AdoHcy (S-adenosyl-L-homocysteine) were 0.1 mM and 0.2 mM, respectively.

** Specific radioactivity of L-(^{3}H)leucine used was 3.98×10^4 dpm/pmol.

*** Endogenous cytochrome *c* was depleted.

All the values were corrected for endogenous values which consisted of either omission of mRNA or addition of protein methylase III heated at 100 °C for 3 minutes. The numbers in the Table represent average of at least two independent determinations with duplicates, and the variations were within 9% from the averages.

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 pI value caused by methylation of apocytochrome *c* at lysine-72[19]. The decrease in pI 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)[40] 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[41]. Furthermore, only methylated cytochrome *c* was extractable from the *poky* mutant of *Neurospora*[42]. 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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