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Effect of Enzymatic Methylation of Proteins on Their Isoelectric Points

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Abstract □ Enzymatic methylation of arginine and lysine residues of several cytochrome *c* and lysine residue of calmodulin always resulted in lowering of their respective isoelectric points (pI). Employing cytochromes *c* derived from various sources, we examined a possible relationship between the degree of amino acid sequence degeneracy and the magnitude of change in the pI values by enzymatic methylation, and found that there was no correlation between these two parameters. By constructing space-filling models of oligopeptide fragments adjacent to the potential methylation sites, we have noted that not all the methylatable residues are able to form hydrogen bonds prior to the methylation. Two preparations of yeast apocytochrome *c*, one chemically prepared by removing heme from holocytochrome *c* and the other by translating yeast iso-1-cytochrome *c* mRNA *in vitro*, exhibited slightly higher Stokes radii than the homologous holocytochrome *c*, indicating relatively "relaxed or open" conformation of the protein. However, when the *in vitro* synthesized methylated apocytochrome *c* was compared with the unmethylated counter-part, the Stokes radius of the latter was found to be larger.

Keywords □ Protein methylation, isoelectric point

During the past decade or so, we have employed enzymatic methylation of cytochrome *c* in Ascomycetes as an experimental model system model system to investigate the biological significance of protein-bound lysine methylation[1-3]. It was earlier discovered that organisms such as *Saccharomyces cerevisiae, Neurospora crassa* and wheat germ contain a highly cytochrome *c*-specific lysine N-methyltransferase (EC 2.1.1.59)[4-7], which methylates *in vitro* a single lysine residue at position 72 of horse heart cytochrome *c*. This position exactly coincides with the *in vivo* methylation site of cytochrome *c* in those organisms[7].

Recently, we observed that the methylation of *in vitro* synthesized yeast 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[8]. Methylation of the apoprotein was accompanied by a large significant change in the isoelectric point of this protein[9,10], possibly indicating a significant structural change of the protein[9,10], possibly indicating a significant structural change of the protein. From space-filling models, we postulated that there is hydrogen bonding between some oxygen and the ϵ-amino group of Res-72 lysine in unmethylated cytochrome *c*, and methylation would cause breakage of any such hydrogen bond. The subsequent conformational change could induce a shift in the "effective charge" of the protein molecule[10]. We further postulated that the yeast mitochondrial receptor for apocytochrome *c* might be signaled by some change in the protein molecule due to its Res-72 lysine methylation[10]. In the present study, we examined several parameters which might be affected by protein methylation. Does methylation of a protein often, or always, bring about a change in pI of the protein? Is there any direct relationship between the breakage of hydrogen bond(s) in protein molecule and the change in the pI induced by methylation? Finally, is there any correlation between the magnitude of pI change by methylation and amino acid sequence variation of each cytochrome *c*?

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MATERIALS AND METHODS

Materials

L-(^{3}H)Leucine (specific activity, 63 Ci/mmol), S-adenosyl-L-(*methyl*-^{3}H)-methionine (specific activity, 74 Ci/mmol), and L-(^{35}S)methionine (specific activity, 140 mCi/mmol) were purchased from Amersham Corp., Arlington Heights, IL. Cytochromes *c* from various sources and myoglobin (whale skeletal muscle) were purchased from Sigma Chemical Co., St. Louis, MO. Ampholine (pH ranges of 3.5-10, 9-11 and 5-8) were obtained from LKB, Sweden. All other chemicals were obtained from various commercial sources and were of the highest purity grade available. S-Adenosyl-L-methionine: cytochrome *c*-lysine N-methyltransferase (protein methylase III; EC 2.1.1.59) was purified from *S. cerevisiae* as described[7]. Unmethylated and enzymatically (*methyl*-^{3}H)-labeled mushroom calmodulin were generous gifts from Dr. Frank L. Siegel, University of Wisconsin, WI.

In vitro methylation of cytochrome c by S-adenosyl-L-methionine:cytochrome c-lysine N-methyltransferase (Protein methylase III)

Cytochromes *c* from various sources (vertebrate) were (*methyl*-^{3}H)-labeled by yeast protein methylase III according to the procedure of DiMaria *et al.*[7]. The final incubation mixture contained 0.9 mg of cytochrome *c* from each source, 0.1 M glycine-NaOH buffer (pH 9.0), 5.7 mM 2-mercaptoethanol, 0.4 mM EDTA, 40μM S-adenosyl-L-(*methyl*-^{3}H)methionine and partially purified yeast protein methylase III (0.7 mg of enzyme protein with specific activity of 21 pmoles of S-adenosyl-L-methionine used/min/mg enzyme protein) in a total volume of 0.25 m*l*. After incubation at 37 °C for 1 hour, the mixture was applied onto a Sephadex G-25 column (0.8 cm × 90 cm) equilibrated with 0.1 M acetic acid to remove unreacted S-adenosyl-L-(*methyl*-^{3}H) methionene. The excluded radioactive fractions were pooled and lyophilized.

In vitro synthesis of yeast iso-1-apocytochrome c and subsequent enzymatic methylation

S. cerevisiae iso-1-apocytochrome *c* labeled with L-(^{3}H)leucine or L-(^{35}S)methionine was synthesized by translating the yeast iso-1-cytochrome *c* mRNA in rabbit reticulocyte lysate *in vitro* translation system as described[8]. The identity of the translation product as yeast apocytochrome *c* was confirmed by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectrofocusing. In order to remove unreacted L-(^{3}H)leucine or L-(^{35}S)-methionine in the translation mixture, the mixture was chromatographed on a Sephadex G-50 minispin column according to the manufacturer's specification (Worthington Biochemicals, Freehold, NJ).

Enzymatically (*methyl*-^{3}H)-labeled yeast iso-1-apocytochrome *c* was prepared by adding protein methylase III together with S-adenosyl-L-(*methyl*-^{3}H)methionine into the translation mixture, as described elsewhere[8]. In this instance, unlabeled leucine or methionine was present in place of radiolabeled amino acids in the above translation mixture, and, thus, iso-1-apocytochrome *c* is obviously labeled with (*methyl*-^{3}H). As before, the unreacted S-adenosyl-L-(*methyl*-^{3}H)-methionine in the incubation mixture was removed by the Sephadex G-50 minispin column technique.

Apocytochrome *c* was also chemically prepared from yeast holocytochrome *c* according to the method of Fisher *et al.*[11]. The amount of protein present in this preparation was determined according to the Coomassie blue binding procedure of Bradford[12].

Isoelectrofocusing chromatography

Isoelectrofocusing (IEF) of various proteins was performed according to the manufacturer's instruction (LKB, Sweden)[13]. A linear gradient of sorbitol was prepared by mixing various volumes of dense and less dense solutions. The dense solution was made of 27 g of sorbitol dissolved in 34.9 m*l* of water and 2.1 m*l* of Ampholine (pH range of 9-11, 20% by w/v). The less dense solution was made of 52.3 m*l* of water and 0.3 m*l* of Ampholine (pH range of 9-11, 20% by w/v), 0.1 m*l* of Ampholine (pH range of 8-10, 20% by w/v), and 0.3 m*l* of Ampholine (pH range of 5-8, 40% by w/v). The gradients were layered upon the densecathode solution which was prepared by dissolving 15 g of sorbitol in 9.5 m*l* of water containing 5.5 m*l* of 1 N NaOH. The anode solution containing 10 m*l* of 0.01 M acetic acid was layered on top of sorbitol gradients. Isoelectrofocusing was carried out in a water-jacket LKB 8100 column (cooled to 2-4 °C) at 800 V for 22-24 hours. Fractions of 0.9 m*l* were collected, and the pH and the absorbance at 280 nm or 550 nm (with cytochromes *c*) of each fraction were measured at room temperature. Radioactivity in a 0.4 m*l* aliquot of each fraction was determined.

Determination of Stokes radius

Th Stokes radii of three types of cytochrome *c*

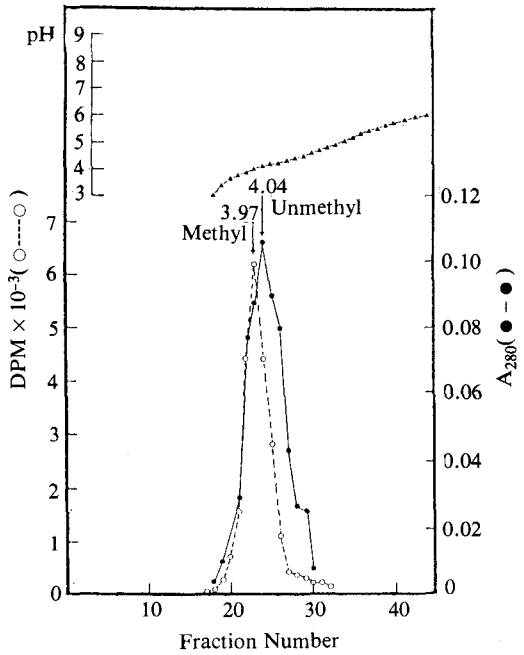

Fig. 1. Isoelectrofocusing chromatography of (*methyl*-^{3}H)-labeled and unmethylated mushroom calmodulin.

Methylated mushroom calmodulin (428,600 dpm) (*methyl*-^{3}H)-labeled by S-adenosyl-L-methionine: calmodulin-lysine N-methyltransferase (EC 2.1.1. 60) and unmethylated counterpart (200 μg) were applied onto the isoelectrofocusing column. Fractions of 1 m*l* were collected, and the pH and the absorbance at 280 nm of each fraction were measured. Unmethylated calmodulin was also measured by the Bradford method[12]. Radioactivity in a 0.1 m*l* aliquot of each fraction was determined. More detailed experimental procedures for isoelectrofocusing are described under Methods.

were determined using a Sephadex G-200 column (1 cm × 90 cm) equilibrated with 0.04 M sodium phosphate, pH 8.0, containing 5 mM EDTA[11,14]. Each was chromatographed separately: either 2.5 mg of chemically prepared yeast apocytochrome *c* or approximately 50,000 dpm of *in vitro* synthesized methylated (*methyl*-^{3}H)-labeled and unmethylated (^{3}H)leucine-labeled yeast apocytochrome *c*. Fractions of 1.6 m*l*/hour were collected, and either the absorbance at 280 nm or the radioactivity of each fraction was determined. The following standards were used; fibrinogen (3 mg), catalase (3 mg), hemoglobin (2.5 mg), bovine serum albumin (3 mg), horse heart cytochrome *c* (2.5 mg) in a total applied volume of 1.0 m*l*.

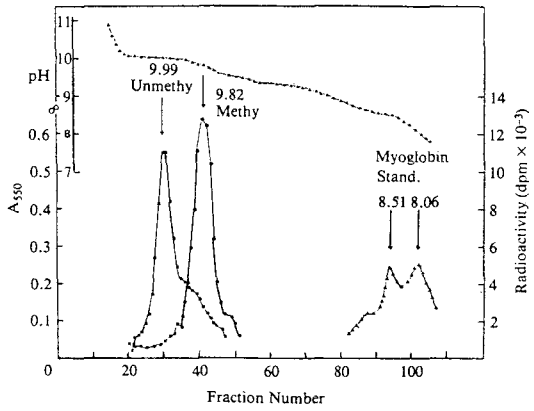

Fig. 2. Isoelectrofocusing chromatography of (*methyl*-^{3}H)-labeled and unmethylated tuna heart cytochrome *c*.

Detailed procedures for (*methyl*-^{3}H)-labelling and isoelectrofocusing of tuna heart cytochrome *c* are described under Methods. Whale skeletal muscle myoglobin, which was used here as internal standard, always showed two protein peaks with different pI values.

RESULTS

Effects of enzymatic methylation of proteins on their isoelectric points

We have earlier observed that enzymatically methylated horse heart cytochrome *c* has a significantly lower pI value than that of unmethylated counter-species[9]. Since methyl substitution of the hydrogen associated with ε-amino group of lysine residue increases the basicity[15,16], the observed decrease in pI value is in direct opposition to the predicted increase. In order to investigate the universality of this observed phenomenon, we examined the possible change of pI value during enzymatic methylation of mushroom calmodulin. Mushroom calmodulin is not methylated *in vivo*[17], thereby providing an excellent experimental model. As shown in Fig. 1, methylation of mushroom calmodulin at Res-115 lysine by S-adenosyl-L-methionine: calmodulin-lysine N-methyltransferase (EC 2.1.1.60)[18] indeed decreased the pI of this portein by 0.07 unit. Although this degree of change of pI is not very large, it is highly significant due to the fact that determination of the pI values of these two species was performed simultaneously. In contrast to the above, the difference in pI values of methylated and unmethylated tuna heart cytochrome *c* is much higher (0.17 unit) (Fig. 2).

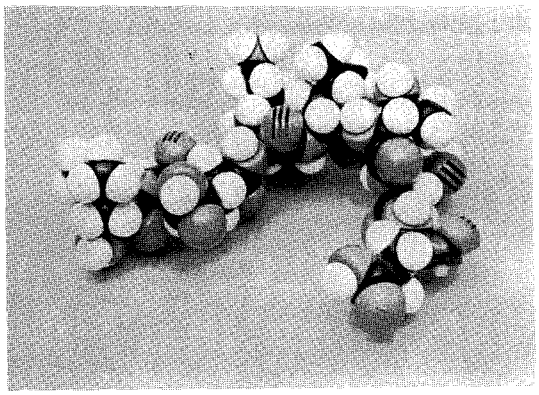

Fig. 3. Space-filling model of residues 112 to 119 of calmodulin.
From left to right: ^{112}Leu-Gly-Glu-Lys-Leu-Thr-Asp-^{119}Glu. The projection at upper left represents Res-115 lysine.

In order to examine the possible coupling of the decrease of pI values with disruption of hydrogen bond at the methylation site, as witnessed during the methylation of horse heart cytochrome *c*[9], a space-filling model of the region surrounding the methylation site of calmodulin (residues between 112 and 119) was constructed (Fig. 3). It is evident from the figure that there is no hydrogen bond formed involving the ε-amino group of Res-115 lysine residue within this region. However, it is quite conceivable that the ε-amino nitrogen of this lysine residue might interact at some distal site due to the tertiary structure of this protein. Unfortunately, the financial restriction of model construction prohibited us from examining this possibility.

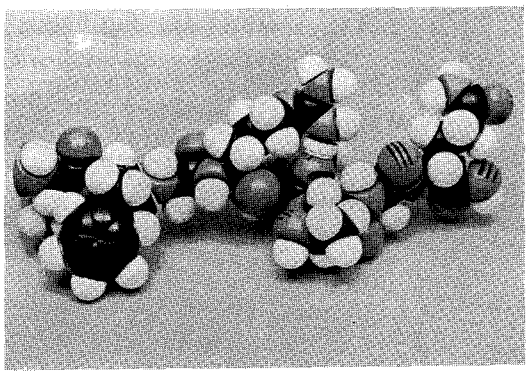

Fig. 4. Space-filling model of horse cytochrome *c* (residues of 35-42).
From ^{35}left to right: Leu-Phe-Gly-Arg-Lys-Thr-Gly-^{42}Gln.
Flat disc near the center indicates the formation of hydrogen bond between the guanidino group of Res-38 arginine and carbonyl group of Res-39 lysine.

We earlier found enzymes in *Euglena gracilis* which methylate Res-38 arginine and Res-65 methionine residues of horse heart cytochrome *c*[19]. The enzymes were highly specific towards arginine and methionine residues, respectively, of *in vivo* unmethylated cytochrome *c*. As in the case of lysine methylation, the pI values of the hemoprotein decreased by 0.70 and 0.80 unit upon methylation of Res-38 and Res-65, respectively (Table I). Therefore, in order to extend the possible correlation between the pI decrease and the inhibition of hydrogen bond formation, a space-filling model of the region of residues 35 to 42 was built (Fig. 4). As seen in

Table I. Effect of enzymatic methylation of protein on its isoelectric point (pI) and possible involvement of hydrogen bond, visualized by space-filling model

Kinds of protein	Source of protein	*In vitro* methylation site	Isoelectric point			Residues in space-filling model	Hydrogen bond	
			unmethyl.	methyl.	difference		observed	formed between residue
MBP*	bovine	107-Arg	n.d.**	n.d.		87-110	+	89-Phe~
Cytochrome *c*	horse heart	38-Arg	10.03	9.33	0.70	35-42	+	~39-Lys
Cytochrome *c*	horse heart	72-Lys	10.03	9.49	-0.54	69-75	+	70-Asn~
Apocytochrome *c*	yeast	72-Lys	9.60	8.70	-0.90	*ibid*	+	70-Asn~
Cytochrome *c*	horse heart	65-Met	10.03	9.23	-0.80	62-69	−	
Calmodulin	mushroom	115-Lys	4.04	3.97	-0.07	112-119	−	
Cytochrome *c****	wheat germ	86-Lys	n.d.	n.d.			−	

* Highly pure unmethylated species are not available at present.
** Not determined.
*** An enzyme which methylates Res-86 lysine is not yet available.

Fig. 4, hydrogen bond could be formed between the nitrogen of guanidino group of Res-38 arginine (methylation site) and oxygen atom of carbonyl of Res-39 lysine.

Table I lists the results of the relationship between the change of pI during enzymatic methylation of lysine, arginine or methionine residues and possible hydrogen bond formation of various proteins, visualized by space-filling models; Res-72 lysine, Res-65 methionine and Res-38 arginine of horse heart cytochrome *c*, Res-115 lysine of calmodulin, Res-86 lysine of wheat germ cytochrome *c*, and Res-107 arginine of bovine myelin basic protein (MBP) were examined. Space-filling models of Res-107 arginine of MBP, and Res-72 lysine and Res-38 arginine of cytochrome *c* demonstrated the formation of hydrogen bond, while Res-115 lysine of calmodulin, Res-65 methionine of horse heart cytochrome *c* and Res-86 lysine of wheat germ cytochrome *c* do not appear to form hydrogen bond with neighbouring amino acids. Therefore, with the limited amount of information available thus far, it could be concluded that there is no direct causal relationship between the decrease of pI values and the breakage of hydrogen bond of proteins during enzymatic methylation.

Effect of amino acid sequence variation on the magnitude of pI change by emzymatic methylation, employing a series of cytochromes c

A question arises concerning how much the degree of variation of amino acid sequence influences the magnitude of pI change resulting from enzymatic methylation. In order to examine this question, we employed a series of cytochromes *c* since it has been well established that, although there are as many as 44 amino acid sequence variations between some cytochromes *c*, their tertiary structures are extremely similar[20,21]. Several cytochromes *c* from a variety of sources were enzymatically methylated, and the pI of both the methylated and unmethylated proteins was compared. As shown in Table II, there was no apparent correlation between the decrease in pI and the variation in the primary sequence.

Effect of enzymatic methylation on Stokes radius

We earlier postulated[8-10] that the enzymatic methylation of protein (cytochrome *c* as an example) results in an alternation of the tertiary structure such that protein acidic groups are more exposed and/or basic groups less exposed, thus effectively making the protein more acidic. Presently, we studied the effect of enzymatic methylation of *in vitro* synthesized yeast apocytochrome *c* on its Stokes radius. Table III lists the Stokes radii of *S. cerevisiae* holocytochrome *c*, and two kinds of yeast apocytochromes *c*: One whose heme was chemically removed, and the other synthesized *in vitro* by translating yeast iso-1-cytochrome *c* mRNA. It is

Table II. Effect of enzymatic methylation on pI values of cytochromes *c* from various sources

Sources of cytochrome *c*	Observed values		difference in pI	variation in amino acid sequence*	pI of unmethylated in literatures
	unmethylated	enzymatically methylated			
Horse heat	10.03	9.49	-0.54	12**	10.65
Chicken heart	9.95	9.70	-0.25	13	10.00
Tuna heart	9.99	9.82	-0.17	21	10.01
Rabbit heart	9.84	9.68	-0.16		10.02
Porcine heart	9.90	9.75	-0.15	10	10.00
Pigeon heart	9.94	9.88	-0.06	13	9.98
Rat heart	9.92	9.88	-0.04		10.03
Canine heart	9.43	9.41	-0.02	10	10.01
Saccharomyces cerev:					
Holocytochrome *c*	9.72	9.68	-0.04	44	10.4
Apocytochrome *c*	9.60	8.70	-0.90	44	

* Number of variations in amino acid sequence is based on human cytochrome *c*.

** Reference 21.

Whale muscle myoglobulin was used as an internal standard and its pI value was 8.25 ± 0.73.

Table III. Stokes radii (A°) of holo- and apocytochrome *c* of *Sccharomyces cerevisiae*

	$K^*_{av.}$	Stokes radius(A°)
Holocytochrome *c*	0.751	18.5
Apocytochrome *c*(heme chemically removed)	0.731 (0.739; 0.722)**	19.0 (19.0; 18.9)
Apocytochrome *c*(*in vitro* translated)	0.724 ± 0.090 (3)***	19.2 ± 1.04

* $K_{av} = \frac{V_e - V_o}{V_t - V_o}$ where V_o represents void volume, V_t total volume and V_e elution volume.
** The numbers in the parentheses are the actual values obtained.
*** Three independent determinations.

evident from the table that apocytochrome *c*, both chemically and *in vitro* synthesized, tends to have a larger Stokes radius than holocytochrome *c*, indicating a more "open" or less "compact" conformation. Holocytochrome *c* is more organized and less reactive with its environment than apocytochrome *c* owing to the 6 coordinate and 2 thioether bonds formed between the heme and apocytochrome *c* molecule[22].

Fig. 5 illustrates Sephadex G-200 column chromatographic determination of Stokes radii of both *in vitro* synthesized methylated [(*methyl*-^{3}H)-labeled] and unmethylated [(^{35}S)methionine-labeled] cytochrome *c*. The difference in the elution volume, or Stokes radii, of these two doubly radiolabeled apocytochrome *c* appears to be rather minimal. However, this small difference is highly significant because chromatographic determination of these two species of apocytochrome *c* was performed simultaneously. According to the result shown in Fig. 5, the methylated apocytochrome *c* has a larger elution volume, thus smaller Stokes radius, than that of the unmethylated protein (see the equation in the legend of Table III). This is indicative of a more organized (or less open) conformation of apocytochrome *c* induced by enzymatic methylation of Res-72 lysine residue.

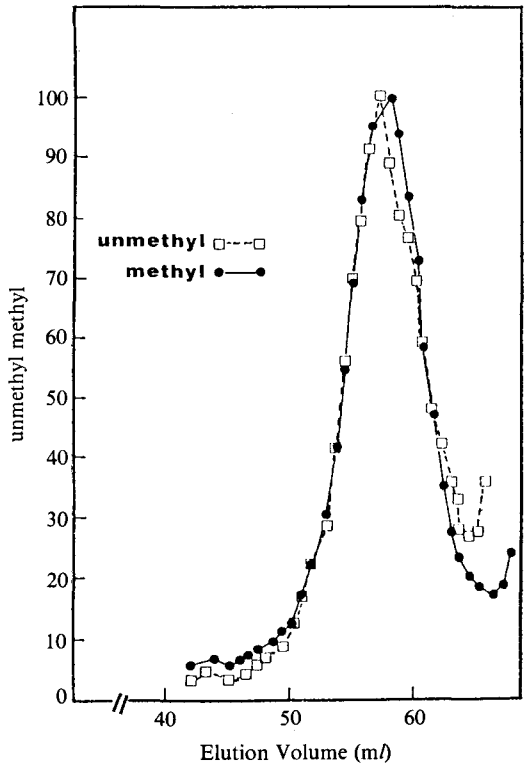

Fig. 5. Sephadex G-200 column chromatography of *in vitro* synthesized methylated and unmethylated yeast apocytochrome *c*.
In vitro synthesized methylated [(*methyl*-^{3}H)-labeled; 74,200 dpm] and unmethylated [(^{35}S)methionine-labeled; 6,900 dpm] yeast apocytochrome *c* in 0.080 ml were applied onto Sephadex G-200 column (1 cm × 90 cm). Fractions of 0.8 ml/30 minutes were collected. Because of the large difference in the amounts of ^{3}H and ^{35}S applied, the result was normalized based on the peak amounts as 100%. The upward curve after the descending is due to some contaminating S-adenosyl-L-(*methyl*-^{3}H)methionine and L-(^{35}S)methionine.

DISCUSSION

The primary purpose of this study is to extend our earlier observation that the enzymatic methylation of Res-72 lysine of *in vitro* synthesized yeast apocytochrome *c* triggers the breakage of hydrogen bond, causing a global conformational alteration of the protein molecule accompanied by lowering the isoelectric point of the protein, to other protein methylation systems. In the case of apocytochrome *c*, this conformational change favours its interaction with a receptor putatively present on the mitochondrial membrane[8].

As shown in Table IV, the effect of enzymatic methylation of the side chains of lysine, arginine and methionine on their isoelectric points is strongly dependent on whether they are in the free or protein-bound states. For example, the pI values of free lysine and ε-N-trimethyllysine are 9.81 and

Table IV. Effect of enzymatic methylation on pI values of free and protein-bound amino acid residues

	pI values			
	free, unmethylated	free, fully methylated	methylated in cytochrome *c*	difference
Lysine	9.81*	10.24*	9.49**	-0.54
Arginine	10.02	11.01***	9.33	-0.70
Methionine	5.71	6.85	9.23	-0.80

* Values obtained from reference 16.
** pI value of unmethylated horse heart cytochrome *c* is 10.03 [9].
*** Enzymatic methylation of side chain nitrogen or sulfur.

10.24, respectively. Therefore, it is expected that enzymatic trimethylation of the ε-amino group of cytochrome *c*-bound lysine residue would increase the pI of the protein by at least 0.43. The experimentally determined value, however, shows a decrease of 0.54 unit in contrast. In the cases of both cytochrome *c*-bound arginine (Res-38) and methionine (Res-65) methylation, the magnitude of the decrease in pI value is much more magnified. This observed phenomenon cannot be explained by change in the ionization in the local environment. Rather, enzymatic methylation would induce a global conformational change of protein, thereby shifting the "effective charge" of the protein molecule and signifying a modification of the tertiary structure such that protein acidic groups are more exposed and/or basic groups less exposed. In support of the above explanation is our earlier observation that the pI values of two hourse heart cytochromes *c* chemically modified at Res-72 lysine with trifluoromethyl-carbamoyl (neutrial group) or carboxydinitrophenyl (acidic group) were affected to a lesser extent than the enzymatically methylated protein[23]. Furthermore, the lowering of the pI value of cytochrome *c* by enzymatic methylation was highly dependent on the higher order structure of the protein; the presence of urea reduced the effect of methylation on the pI value with the difference virtually disappearing with the increasing concentration of urea to 6 M, which essentially disrupts all secondary and tertiary structure of protein.

The lowering of the pI values of proteins by enzymatic methylation appears to be universal in protein methylation reactions (Table I, II and IV). This effect occurs in proteins such as calmodulin and cytochromes *c* from variety of sources, and with amino acid residues such as lysine, arginine and methionine. Furthermore, the conclusion derived from the result in Table II is that degree of pI change by enzymatic protein methylation is not directly correlated with the extent of amino acid variations. Rather, it appears to be closely related to tertiary structures of the proteins, which becomes more evident from the following considerations. Despite large sequence variations among the wide range of eukaryotic species[21], cytochrome *c* has retained a remarkable degree of consistency in biological function, tertiary structure, and a number of other important properties including redox potential (invariably ~ 250 mV)[20]. Furthermore, we earlier observed that enzymatic methylation of *in vitro* synthesized yeast apocytochrome *c* had much more pronounced effect on lowering the pI than with holocytochrome *c*[10]. It is known that the tertiary structure of holocytochrome *c* is more rigid due to 2 thioether and 6 coordinate bonds formed between the prosthetic group and apocytochrome *c* molecule[22]

In our earlier hypothesis concerning the biochemical function of apocytochrome *c* methylation, we suggested that the initial trigger to enhance its import into mitochondria was the disruption of a hydrogen bond formed presumably between Res-70 asparagine and Res-72 lysine residues[8-10], demonstrated by building space-filling models. Extending this observation in this paper, the space-filling models of limited lengths of peptides containing *in vivo* methylation sites (Fig. 3, 4 and unpublished data) demonstrated the possible formation of hydrogen bonds at Res-107 arginine of myelin basic protein (MBP) and Res-38 arginine of cytochrome *c* in addition to Res-72 lysine of this protein (Table I). On the other hand, no hydrogen bond formation was observed in the models including Res-65 methionine and Res-86 lysine of cytochrome *c*, and Res-115 lysine of calmodulin. Thus, it seems that the disruption of the hydrogen bond during cytochrome *c* methylation is a special case rather than common phenomenon. On the other hand, it is quite possible that S of Res-65 and N of Res-86 and of Res-115 might form hydrogen bonds with some residues distal in the sequence but present in close proximity in the globular tertiary structure.

Although many biochemical differences between the methylated and unmethylated cytochrome *c* (and apocytochrome *c*) were observed [such as large accumulation of methylated species in *poky* mutant *N. crassa* mitochondria[24] and more efficient binding of the methylated cytochrome *c* to yeast mito-

chondria[1,8]], studies on the physicochemical properties of these two forms of cytochrome *c* revealed no significant difference between them. Polastro and his coworkers found that the heme environment and its coordination sphere were not affected by the methylation state of the protein[25,26], and Scott and Mitchell[24] observed similar sedimentation coefficients and U.V. and visible absorption spectra with these two protein species. It was, therefore, to our great surprise to observe that *in vitro* synthesized methylated yeast apocytochrome *c* eluted more slowly than the unmethylated species on Sephadex G-200 column (Fig. 5). The result in Fig. 5 indicates that Stokes radius of the methylated species is smaller than that of the unmethylated. The observed decrease of Stokes radius might suggest that the structure of apocytochrome *c* becomes more "compact" upon enzymatic methylation such that mitochondrial receptor for apocytochrome *c* recognizes this protein more easily than the unmethylated apocytochrome *c*.

In conclusion, one of the most notable general characteristics of protein-bound N- and S-methylation is the lowering of the isoelectric point (pI) of the protein. Contrary to our earlier assumption, however, disruption of hydrogen bond might not necessarily be involved as an initial "trigger" process. In order to explain the more efficient import of methylated apocytochrome *c* into mitochondria, we put forward a hypothesis that altered conformation of apocytochrome *c* induced by enzymatic methylation would favour its interaction with the receptor on mitochondrial membrane. However, this scenario of events might not be applicable in all of the protein-bound lysine methylation. For example, methylation of protein-bound lysine residues was found to provide a metabolic precursor for carnitine biosynthesis[27], enzymatic methylation of Res-56 lysine of elongation factor EF-Tu in *Escherichia coli* decreases its rate of tRNA-dependent GTP hydrolysis[28], and methylation of Res-115 lysine of calmodulin lowers its NAD-kinase activity[29]. Of course, these proteins are not transported to specific intracellular sites, as is cytochrome *c*, so the need for secondary and tertiary structural alterations is not evident.

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