Despite this broad specificity, substrates are hydrolyzed only at selective peptide bonds. This selectivity may be due to the influence of neighboring groups or to the conformation of the substrate. For example, hydrolysis of LH-RH at the Trp^3–Ser^4 and Leu^7–Arg^8 peptide bonds is not observed, and as shown for various LH-RH analogues (Horsthemke et al., 1981), the rate of hydrolysis of a given peptide bond is strongly influenced by modifications of the substrate even at positions remote from the scissile peptide bond. In addition, we have found that the LH-RH (1–9) nonapeptide is degraded 50 times more rapidly than LH-RH itself (data not shown). Further studies are necessary to characterize the determinants for such effects, which are typical for an endopeptidase with an extended substrate-binding region.

Although the enzyme was discovered when the degradation of LH-RH was investigated, it is obviously not an LH-RH specific peptidase. Therefore, its possible physiological function as a neuropeptide-degrading enzyme warrants further investigations. The determination of the cleavage specificity presented in this report may help to synthesize enzyme-specific substrates and inhibitors suitable for further studies.

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Cytochrome *c* Specific Methylase from Wheat Germ†

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ABSTRACT: The cytochromes *c* of plants (e.g., wheat germ) possess two trimethyllysines, residues 72 and 86. In order to investigate the nature of these methylations, we have purified a cytochrome *c* specific methylase *S*-adenosylmethionine: protein(lysine) *N*-methyltransferase (protein methylase III) from wheat germ 135-fold. The in vitro site of methylation by both the purified enzyme and crude wheat germ extract toward various forms of horse heart cytochrome *c* was localized by two-dimensional peptide mapping, Aminex A-5 column peptide analysis, and CNBr cleavage analysis to be the residue 72 lysine. However, no additional sites, in particular residue 86, were seen to be methylated. Although the enzyme is highly specific toward cytochrome *c* as an in vitro protein substrate, avian cytochromes *c* are seen to be much better substrates than those from mammalian sources. The enzyme possesses an extremely low K_m for apocytochrome *c* (1.21 μM), suggesting that methylation may occur before heme attachment in vivo. Various *S*-adenosyl-L-homocysteine analogues were tested for their inhibitory capability toward the enzyme; it was observed that only the D and L forms of *S*-adenosylhomocysteine are inhibitors while analogues modified in the adenine or homocysteine moieties do not possess inhibitory capability. Results from the Aminex A-5 column chromatography of horse heart cytochrome *c* chymotryptic digest showed the N^{ϵ}-methyl-, N^{ϵ}-dimethyl-, and N^{ϵ}-trimethyllysine forms of the residue 68–74 peptide to elute earlier than the unmethylated form. This result suggests that the methylated peptides are less basic than the unmethylated form.

The N^{ϵ}-methylated lysines have been shown to occur in a wide variety of proteins from many species of organisms (Paik & Kim, 1971, 1975, 1980). In particular, the cytochromes *c* of fungal, plant, and protozoan sources contain residues of trimethyllysine while animal cytochromes *c* lack this amino acid (Paik & Kim, 1980).

Much work has been done in seeking to understand the nature and function of cytochrome *c* methylation, particularly in fungal organisms. The methylases that catalyze the reaction were purified from two fungal sources, *Neurospora crassa* and baker's yeast, and were found to be highly specific in regard to their exclusive recognition of cytochrome *c* as a substrate (Durban et al., 1978; DiMaria et al., 1979). In addition, they exhibit a further specificity in that they recognize only the residue 72 lysine as a site of methylation when horse heart cytochrome *c* is used as a substrate (DiMaria et al., 1979). This observation correlates well with the in vivo occurrence of trimethyllysine at exclusively the residue 72 in the cytochromes *c* of *N. crassa* and baker's yeast (DeLange et al., 1969, 1970).

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In relation to a possible function, methylation increases the affinity of the cytochrome *c* to yeast mitochondria (Polastro et al., 1978) and, causally related to this, stabilizes the molecule to intracellular proteolytic breakdown (Farooqui et al., 1981). Moreover, in direct opposition to the fact that it renders the lysine more basic, methylation has been shown to significantly diminish the basic character of the molecule as demonstrated by methylated cytochrome *c*'s behavior on cation-exchange resins (Scott & Mitchell, 1969) and isoelectric-focusing columns (Kim et al., 1980). As a consequence of these observations, it has been suggested that methylation may have a profound effect in that it could enable acidic residues to become more exposed as a result of a conformational change in the molecule (Kim et al., 1980).

Although this work is indicative of the well-understood nature of cytochrome *c* methylation in fungal organisms, little has been done in the study of this methylation in plants. The cytochromes *c* in virtually every plant species studied have been found to possess two trimethylated lysine residues, residues 72 and 86 (Paik & Kim, 1980). This situation contrasts sharply with the single residue 72 trimethyllysine found in fungi and therefore raises questions about the nature and function of cytochrome *c* methylation in plants. In the present study, we have addressed certain aspects of the plant cytochrome *c* methylation problem. Specifically, the enzymology of cytochrome *c* methylation in wheat germ is considered.

Materials and Methods

Materials. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine (sp act. 60.6 μCi/μmol) was obtained from Amersham. Pigeon and chicken cytochromes *c* were purified as described by Margoliash & Walasek (1967). The other cytochromes *c*, horse heart (type VI), dog, pig heart, *Candida krusei*, and yeast, as well as the noncytochrome *c* proteins used were obtained from Sigma. Also from Sigma were tosyllysine chloromethyl ketone treated α-chymotrypsin (type VII), calcium phosphate gel, glycine, and cyanogen bromide. Sephadex G-25 and G-50 and DEAE-Sephacel were obtained from Pharmacia. Aminex A-5 resin was from Bio-Rad. Throughout the study, the scintillation mixture Formula 963 from New England Nuclear was used. *S*-Adenosyl-L-homocysteine and its analogues, *S*-adenosyl-D-homocysteine, *S*-adenosyl-DL-homocysteine, *n*-butylthioadenosine, and (hydroxyethyl)thioadenosine, were obtained from Sefochem Fine Chemical Ltd. (Israel). *S*-Inosyl-L-homocysteine and methylthioadenosine were prepared as previously described (Oliva et al., 1980). All other reagents were of the highest grade obtainable.

Enzymatic Assay. The routine assay of the enzymatic methylation of horse heart cytochrome *c* was carried out by the procedure previously described (Durban et al., 1978) in a final volume of 0.25 mL. The final assay mixture contained 0.1 M glycine–NaOH (pH 9.0), 5.7 mM mercaptoethanol, 1 mg of horse heart cytochrome *c* (native or denatured), 33 μM AdoMet[1] (103 cpm/pmol), and in most cases 80 mM KCl.

Analysis of Amino Acids. Peptide or protein samples were hydrolyzed in 6 N HCl for 48 h at 110 °C in vacuo. Conventional total amino acid analysis (acidic–neutral and basic) was carried out by the method previously described (Spackman et al., 1958). In the cases where specific resolution and quantitation of radioactive N^{ϵ}-methyl-, N^{ϵ}-dimethyl-, and N^{ϵ}-trimethyllysine was necessary, basic amino acid analysis was performed as described by Paik & Kim (1967). Radioactivity was monitored with a flow cell in a Packard Tri-Carb Model 2002 liquid scintillation spectrometer with approximately 65% efficiency.

Preparation of Modified Cytochromes c and Peptides. Ethanol-denatured cytochrome *c* was prepared by the method described by Margoliash et al. (1962). Apocytochrome *c* was prepared similar to the method described by Fisher et al. (1973). The residue 1–65 CNBr peptide was prepared from apocytochrome *c* by the previously described procedure (Durban et al., 1978).

Chymotryptic Digestion and Peptide Mapping. Enzymatic methylation of cytochrome *c* for chymotryptic peptide analysis was carried out as described specifically in the text. The radiolabeled cytochrome *c* was separated from the unreacted radiolabeled AdoMet by chromatography on a Sephadex G-25 column (1 × 30 cm) that was equilibrated in 10 mM ammonium bicarbonate. The excluded protein peak was lyophilized and then subjected to chymotryptic digestion as described by Brautigan et al. (1978). Specifically, the protein was dissolved to a concentration of 10 mg/mL in 0.1 M ammonium bicarbonate. To this was added tosyllysine chloromethyl ketone treated α-chymotrypsin to a final concentration that was 5% (w/w) with the substrate protein. The digestion was allowed to proceed for 20 h at 28 °C. At the end of the incubation, the reaction mixture was frozen and lyophilized 3 times in order to remove the ammonium bicarbonate. The lyophilized peptides were dissolved in a minimal amount of electrophoresis buffer [water–pyridine–acetic acid (1800:200:7)] and then applied to Whatman 3 MM paper for peptide mapping. The solvents and running conditions for the two-dimensional mapping procedure have been described previously (DiMaria et al., 1979).

CNBr Cleavage and Separation of the CNBr Peptides. After enzymatic methylation of cytochrome *c* as described in the text, the hemoprotein (6 mg) was precipitated by the addition of 7.5% trichloroacetic acid, and the unreacted AdoMet was removed by three trichloroacetic acid washings, followed by two absolute ethanol washings to remove residual trichloroacetic acid. After allowing it to air-dry, the protein was cleaved with CNBr by the method described (Durban et al., 1978). Briefly, the protein was suspended in 10 mL of 0.1 N HCl containing 5 mg/mL CNBr. The reaction was carried out in the dark for 40 h at 30 °C with shaking. The reaction mixture was then lyophilized and suspended in 0.2 mL of 10% formic acid. This was applied to a Sephadex G-50 column (0.9 × 110 cm), which was equilibrated and run in 10% formic acid. Fractions of 0.5 mL were collected and monitored for A_{280} absorbance, and radioactivity was determined on 100-μL aliquots.

Aminex A-5 Chromatography. Chymotryptic digests of enzymatically methylated apocytochrome *c* were chromatographed on an Aminex A-5 column (0.9 × 60 cm) equilibrated with 0.2 M pyridine acetate buffer (pH 3.1) in a way similar to the previously described procedure (DiMaria et al., 1979). After sample application, the column was eluted with a linear gradient of the equilibration buffer and 2 M pyridine acetate (pH 5.0). The total volume of the gradient was 1.5 L. The flow rate was 30 mL/h and 2.5-mL fractions were collected. Ninhydrin coloration was determined on 100-μL aliquots as described (Hirs et al., 1956) while radioactivity was monitored on 50-μL aliquots. The peptide-containing peaks as determined by ninhydrin were pooled, lyophilized to dryness, and further purified by preparative descending chromatography on Whatman 3 MM paper sheets with 1-butanol–pyridine–acetic

[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosylhomocysteine, D or L form as specified; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DEAE, diethylaminoethyl.

acid–water (60:40:12:48) as a solvent system (Brautigan et al., 1978). The peptides were eluted and concentrated by the method previously described (Kasper, 1975).

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951).

Results

Purification of Wheat Germ Cytochrome c Methylase. Wheat germ, which was obtained from General Mills, was stored desiccated at 0–4 °C until use. All purification manipulations were carried out at 0–4 °C. Dry wheat germ flake (20 g) was suspended in 5 volumes (100 mL) of 20 mM sodium phosphate buffer (pH 7.0) containing 14 mM mercaptoethanol and magnetically stirred for about 2 h. This procedure was shown to be sufficient to solubilize virtually all of the measurable cytochrome *c* methylase activity. The thick homogenate was centrifuged at 39000*g* for 30 min, and the supernatant (83.4 mL) was then made 42% saturated in ammonium sulfate by the dropwise addition with stirring of an appropriate amount of the salt solution that was saturated at room temperature. After about 2 h, the precipitate was collected by centrifugation at 39000*g* for 10 min. The precipitate was subsequently resuspended in 83 mL of 5 mM sodium phosphate buffer (pH 7.0) containing 14 mM mercaptoethanol (buffer A) to give a final volume of 87 mL. This was then subjected to calcium phosphate gel treatment by the addition of 2 volumes of the gel suspension, which contained 17 mg of solids/mL. The protein was permitted to adsorb to the gel for 2 h with occasional stirring. The gel was collected by centrifugation at 700*g* for 5 min, and the resulting pellets were washed with buffer A. The washed gel was eluted with 50 mL of 0.1 M sodium phosphate (pH 7.0) containing 14 mM mercaptoethanol and then centrifuged at 39000*g* for 10 min. The supernatant (50 mL) was made 50% saturated in ammonium sulfate by the addition of an equal volume of the saturated solution. At this stage, the enzyme can be stored for at least 1 week without apparent loss in activity. The ammonium sulfate suspension (100 mL) was centrifuged at 39000*g* for 10 min and then resuspended in 10 mL of 10 mM Tris-HCl (pH 7.3) containing 0.5 mM EDTA, 20% glycerol, and 14 mM mercaptoethanol (buffer B). The residual amounts of ammonium sulfate were removed from this clear suspension by passing it over a Sephadex G-25 column (2 × 30 cm) equilibrated in buffer B. The excluded protein peak was collected and charged to a DEAE-Sephacel column (1 × 10 cm) equilibrated with buffer B. The conditions of elution as well as the elution profile in the region of demonstrated enzyme activity are given in Figure 1. The enzyme activity profile shows two activity peaks exist: one centered at fraction 51 and the other centered at fraction 68. The latter peak, which is obviously the major one, was positively identified to contain cytochrome *c* methylating activity by product analysis that showed the presence of methylated lysines. The former and smaller of the two peaks was found not to represent cytochrome *c* dependent activity but rather was an uncharacterized endogenous activity, which was not investigated further. The major activity peak was pooled (27 mL) and was concentrated with an immersible CX-10 ultrafiltration unit (Millipore). The cytochrome *c* methylase activity is seen to elute at 0.205 M KCl (Figure 1). The elution behavior is similar to that of the yeast cytochrome *c* methylase when run under similar conditions on DEAE-cellulose (data not shown). In Table I, the purification has been summarized. It is seen that the enzyme is 135-fold purified with a yield of 22%.

Enzyme Stability and Some General Properties. The methylating activity of the DEAE-purified enzyme is linearly dependent on the amount of added enzyme protein and the time of assay incubation (data not shown). Under assay conditions, the enzyme stability is very much enhanced by the presence of mercaptoethanol (5.8 mM) and to a lesser extent by the inclusion of KCl (80 mM). Because of this, these components were routinely added to the assay mixture in most of the studies done with the enzyme. With regard to long-term stability, the activity of the enzyme over the course of storage at 4 °C in 10 mM Tris-HCl (pH 7.3) containing 0.5 mM EDTA, 20% glycerol, and 2.8 mM mercaptoethanol was determined. After 26 days, only 16% of the initial activity remained. However, when this relatively inactive enzyme preparation was made 70 mM in mercaptoethanol and incu-

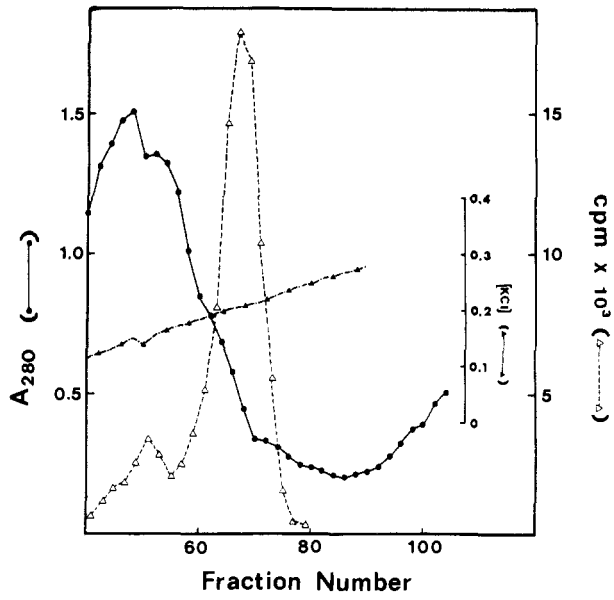

FIGURE 1: DEAE-cellulose chromatography of protein methylase III from wheat germ. Calcium phosphate gel eluate treated as described in the text was charged to a DEAE-Sephacel column (1 × 10 cm) equilibrated with buffer B (see text). After elution of the unbound protein with the same buffer, the column was eluted with a linear KCl gradient in buffer B to 0.5 M (total volume of the gradient was 200 mL). 1.7-mL fractions were collected with the flow rate at 12 mL/h. Enzyme activity measurements were performed on 50-μL aliquots from the fractions with 1 mg of denatured horse heart cytochrome *c* in the assays.

Table I: Purification of Cytochrome *c* Specific Protein Methylase III from Wheat Germ

purification step	vol (mL)	protein		enzyme activity[a]		*x*-fold purification	yield (%)
		mg/mL	total mg	sp act.[b]	total act.[c]		
homogenate	118.0	53	6254	3.75	23 452	1.0	100
supernatant at 39000*g*	83.4	30.2	2519	7.38	18 590	2.0	79.3
$(NH_4)_2SO_4$ precipitate	86.7	13.9	1205	14.45	17 712	3.9	74.2
calcium phosphate gel	50.0	3.32	166	55.25	9 171	14.7	39.1
$(NH_4)_2SO_4$ precipitate	20.0	7.60	152	60.14	9 126	16.0	39.1
DEAE column chromatography	27.0	0.37	10	507.8	5 078	135.4	21.7

[a] Enzyme activity was measured by the procedure described under Materials and Methods with 1 mg of denatured horse heart cytochrome *c* as a protein substrate. [b] Specific activity is expressed as pmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine used min^{-1} (mg of enzyme protein)$^{-1}$. [c] Total activity is expressed in pmol of AdoMet used min^{-1}.

Table II: Protein Substrate Specificity[a]

protein substrate and source	pmol of incorpn/ 20-min incubn	% relative to native horse heart cytochrome *c*
cytochrome *c*		
horse heart (native)	7.71	100
horse heart (denatured)	198.4	2573.0
pigeon	115.4	1497.0
chicken	60.8	788.0
canine	13.1	173.0
pig heart	13.4	174.0
Candida krusei[b]	5.15	67.0
Saccharomyces cerevisiae[b]	0.93	12.1
histone type II (Sigma)	1.19	15.5
histone type III (Sigma)	0.46	6.0
serum albumin (bovine)	0	0
myoglobin (sperm whale)	0.32	4.2
ribonuclease (bovine pancreas)	0.22	2.9
trypsin inhibitor (soy bean)	0.15	1.9
hemoglobin (bovine)	0.56	7.3
protamine sulfate (herring)	0.73	9.5
poly-L-lysine ($M_r > 70\,000$)	0	0
γ-globulin (bovine)	0.05	0.6

[a] 31.5 μg of the DEAE-purified wheat germ enzyme along with 1 mg of the appropriate protein substrate was used in the incubations (20 min) carried out as described under Materials and Methods. [b] Contains 1 mol of trimethyllysine/mol of hemoprotein (DeLange et al., 1970).

Table III: Substrate Capability of Variously Treated Cytochromes *c*[a]

source and type of treatment	crude enzyme: pmol of AdoMet/ 10-min incubn	crude enzyme: relative activity	DEAE-purified enzyme: K_m (μM)	DEAE-purified enzyme: V_{max}[b]
horse heart				
native	9.3	1.00	6670	133.0
denatured	47.8	5.14	12.9	487.8
apocytochrome *c*	66.5	7.15	1.21	540.5
aporesidue 1–65	nd[c]	nd	78.0	142.9
yeast				
native	3.8	1.00	nd	nd
denatured	1.37	0.36	nd	nd
apocytochrome *c*	0.17	0.26	nd	nd

[a] Kinetic constants (K_m and V_{max}) for the DEAE-purified enzyme were determined with 19.6 μg of enzyme protein in the assays. For the assays utilizing the crude enzyme, 0.75 mg of wheat germ supernatant was used as an enzyme source with 1 mg of native or ethanol-denatured cytochromes *c* or 0.12 mg of the apocytochromes *c*. [b] V_{max} is expressed as pmol incorporated min^{-1} (mg of enzyme protein)$^{-1}$.

bated at room temperature for 1 h, full activity was restored. In light of these observations, the enzyme was routinely stored in the presence of 70 mM mercaptoethanol.

The K_m for AdoMet was determined to be 47.6 μM. This K_m value is of the same general magnitude as that determined for the *Neurospora* and yeast cytochrome *c* methylases [19 and 40 μM, respectively (Durban et al., 1978; DiMaria et al., 1979)].

Substrate Specificity. The partially purified enzyme was assessed for its substrate specificity toward various proteins (Table II). Among a wide variety of proteins tested, only the various cytochromes *c* (excluding yeast) served as substrates. Significantly, among the nonsubstrates were the histone II and III preparations. Although found to be substrates for the mammalian protein methylase III (Paik & Kim, 1970), these proteins were also nonsubstrates in the cases of the other cytochrome *c* methylases from *Neurospora* and yeast (Durban et al., 1978; DiMaria et al., 1979).

In comparing the relative substrate capability of the native cytochromes *c*, a remarkable amount of variation is evident: from 67% for *Candida* to 1497% for pigeon cytochrome *c*. The avian cytochromes *c*, pigeon and chicken, are much better substrates than the mammalian cytochromes *c*, horse, pig, and dog. This sharp discrimination with respect to species origin of the cytochrome *c* was not exhibited by the *Neurospora* and yeast cytochrome *c* methylases (Durban et al., 1978; DiMaria et al., 1979). While it can be envisioned that some amino acid sequence difference is ultimately responsible for the increased substrate capability of the avian cytochrome *c*, there is an indication that this may not be necessarily so. As shown in Table II, when one of the poorest substrates, horse heart cytochrome *c*, is ethanol denatured, its substrate capability increases 25-fold. In this case, a disruption in tertiary structure appears to best explain the increase in substrate activity.

Although both yeast and *Candida* cytochromes *c* possess 1 mol of trimethyllysine per mol of hemoprotein in vivo (DeLange et al., 1970), they differ in the fact that the *Candida* protein is seen to be a substrate (67%) while the yeast species is not. The failure of the yeast cytochrome *c* to be methylated is consistent with the fact that it has been unequivocally shown to be in vivo methylated at residue 72. It has also been supposed that *Candida* is similarly in vivo methylated at this position, but this has never been ascertained experimentally. Indeed, the observed substrate capability indicates that *Candida* cytochrome *c*'s complement of trimethyllysine most probably resides whole or at least in part at a lysine other than residue 72. A further indication of this has also been shown by comparison of peptide maps of *Candida* and horse heart cytochromes *c* both in vitro methylated by *N. crassa* cytochrome *c* methylase. In these studies, the *Candida* species is seen to be in vitro methylated at the same site as that of horse heart (previously shown to be residue 72) (data not shown).

Substrate Capability of Variously Treated Cytochromes c. The effect of various treatments on cytochrome *c*'s ability to act as a substrate toward the wheat germ enzyme in the crude and DEAE-purified states is shown in Table III. In the case of the crude enzyme, denatured and apo horse heart cytochromes *c* are seen to be much better substrates than the respective native hemoprotein, 5.14- and 7.15-fold increase, respectively. This trend is even more strikingly evident when the kinetic parameters determined with the DEAE-purified enzyme are compared for the various substrates. First, the native cytochrome *c* is a relatively poor substrate; K_m is 6670 μM and V_{max} is 133 units/mg. However, denaturation of the cytochrome *c* lowered the K_m and raised the V_{max} to values of 12.9 μM and 487 units/mg, respectively. Removal of the heme prosthetic group even further enhances substrate capability in that the K_m of apocytochrome *c* is only 1.21 μM with a V_{max} of 540.5 units/mg. The apocytochrome *c* residue 1–65 CNBr fragment despite its lack of methylation sites seen in vivo is also a fairly good substrate with a K_m of 78 μM.

The substrate capability of variously treated yeast cytochromes *c* was also examined with the crude enzyme as is shown in the lower portion of Table III. It is evident that ethanol denaturation and heme removal do not have the effect of improving the apparently poor substrate capability of the native molecule. Although the native yeast cytochrome *c* seems to have a limited amount of substrate capability, an analysis of the radioactive methylated amino acids in the enzymatic reaction product revealed that the observed radioactivity was not methylated lysines as was the case for horse heart cytochrome *c* but rather was an uncharacterized acid-labile

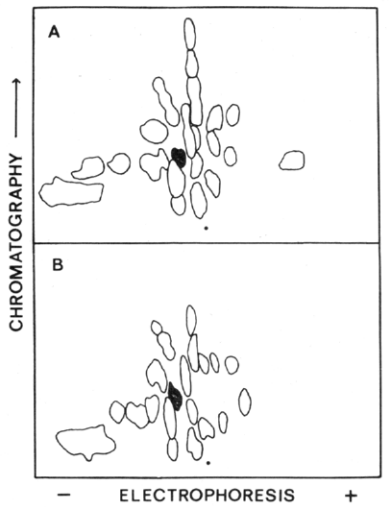

FIGURE 2: Peptide mapping of chymotryptic digests of native horse heart [*methyl*-^{14}C]cytchrome *c*. The peptide maps for cytochrome *c* methylated by the wheat germ DEAE-purified enzyme (panel A) or the purified yeast enzyme (panel B). For the wheat germ enzyme, 10 mg of native horse heart cytochrome *c* was methylated with 125 μg of the enzyme in a final volume of 1.5 mL, using the assay conditions described under Materials and Methods for an incubation period of 1 h. Native horse heart cytochrome *c* was similarly methylated with the yeast enzyme previously described (DiMaria et al., 1979). The samples were further treated as described under Materials and Methods. The black dots are the point of application of the digests. Electrophoresis in the horizontal dimension and chromatography in the vertical dimension were performed sequentially. Outlined areas, ninhydrin positive spots; solid areas, radioactive as shown by autoradiography.

product. Thus, the cytochrome *c* specific methylase(s) in the crude enzyme preparation can recognize only the various forms of horse heart cytochrome *c* as substrates, suggesting that the enzyme activity is specific toward the residue 72; i.e., when the residue 72 site is blocked as in the case of yeast cytochrome *c*, all substrate activity is abolished.

Site Specificity of Wheat Germ Cytochrome c Methylase. The problem of amino acid residue specificity for the wheat germ enzyme is particularly interesting because of the fact that plant cytochromes *c* possess two methylated lysine residues (residues 72 and 86). In order to ascertain the site specificity of the partially purified methylase, we subjected chymotryptic digests of horse heart cytochrome *c* that was enzymatically methylated in vitro to peptide mapping. The results are shown in Figure 2. In panel A, the peptide map for the wheat germ enzyme methylation product is given. For comparison, in panel B, the yeast-enzyme methylation product is shown. The peptides as visualized by ninhydrin are outlined, and those that are radioactive are shaded. It is clear that the wheat germ enzyme methylation product is similar to the yeast-enzyme product in two respects. For one, the wheat germ product shows only one radioactive peptide. Furthermore, this peptide corresponds well in its two-dimensional mobility to the radioactive peptide in the case of the yeast enzyme (panel B). Because the radioactive peptide in the case of the yeast enzyme has been previously identified as the residue 68–74 peptide of cytochrome *c* (DiMaria et al., 1979), the present data show the wheat germ enzyme site of methylation to reside on the same peptide, most likely at the residue 72 although residue 73 cannot be ruled out. This result is consistent with residue 72 being methylated in vivo in wheat germ cytochrome *c* (DeLange et al., 1969). However, because residue 86 is also in vivo methylated, it was surprising that an additional radioactive peptide presumably containing the residue 86 was not also seen.

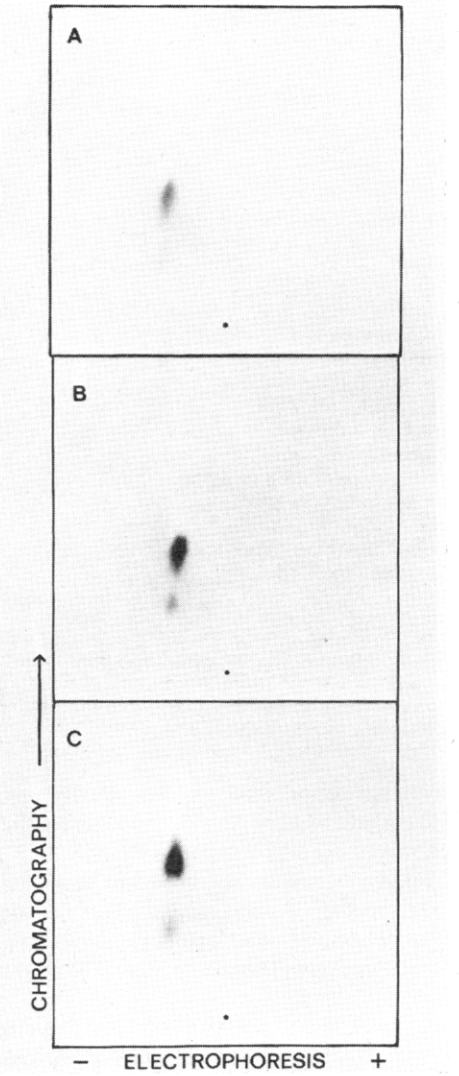

FIGURE 3: Peptide mapping of chymotryptic digests of wheat germ enzyme-methylated apocytochrome *c* or ethanol-denatured horse heart cytochrome *c* and comparison with yeast enzyme-methylated apocytochrome *c*. Panels A and B are ethanol-denatured cytochrome *c* and apocytochrome *c*, respectively, both methylated with wheat germ cytochrome *c* methylase. In these cases, 0.12 mg of apocytochrome *c* or 0.5 mg of denatured horse heart cytochrome *c* was in vitro methylated with 356 μg of DEAE-purified enzyme in a final volume of 2.5 mL for an incubation of 1 h. After incubation, 1 mg of apocytochrome *c* was added to the methylated apocytochrome *c* as carrier. The samples were further prepared for peptide mapping as described under Materials and Methods. Panel C is apocytochrome *c* methylated with the DEAE-purified yeast enzyme (DiMaria et al., 1979) similar to the method above. This sample was similarly prepared for peptide mapping. The black dots are the points of application of the digests. Electrophoresis in the horizontal and chromatography in the vertical dimension were performed sequentially. Dark areas represent exposed areas on the X-ray autoradiograms of the peptide maps.

Since native cytochrome *c* is a poor substrate for the wheat germ enzyme (see Table III), the much better substrates, ethanol-denatured cytochrome *c* and apocytochrome *c*, were examined for the possibility that by virtue of their high substrate capability, they may contain additional methylation site(s). These modified cytochrome *c* species were methylated in vitro, chymotryptically digested, and run on peptide maps in the way just described for the native cytochrome *c*. In Figure 3, the peptide maps for ethanol-denatured cytochrome *c* and apocytochrome *c* methylated by wheat germ enzyme are given in panels A and B, respectively. For comparison apocytochrome *c* that was methylated by the purified yeast en-

zyme is given in panel C. In all three cases, a similar pattern of radioactive peptides is observed. That is, there are two spots evident: one of the higher chromatographic mobility that is very intense and a much less intense radiolabeled peptide that is less mobile chromatographically. Although the existence of these two spots in the case of the wheat germ enzyme methylation product could imply a multiplicity of methylation sites, this conclusion is untenable in lieu of the fact that the same result is also seen in the case of the yeast enzyme. Indeed, the yeast enzyme has been previously shown to have the same unique site specificity toward apocytochrome *c* as that determined for native cytochrome *c* (DiMaria et al., 1979). The existence of the two radioactive spots is easily explained by the fact that chymotryptic digestion of horse heart cytochrome *c* is known not only to generate the residue 68–74 peptide but also a minor amount of a similar peptide lacking the residue 68 leucine (residue 69–74 peptide) (Margoliash, 1962). This shorter peptide has been shown to be similar in electrophoretic mobility but slower chromatographically then the residue 68–74 peptide (Margoliash & Smith, 1962).

The major radioactive chymotryptic peptide from apocytochrome *c* methylated by wheat germ enzyme was ascertained to be residue 72 containing peptide (residue 68–74) by the following analysis of the chymotryptic peptides. The peptides were first separated by Aminex A-5 chromatography with the chromatographic elution profile in the region of the radioactive peptides being given in the upper portion of Figure 4. The ninhydrin-positive peaks were pooled, concentrated, and purified as described under Materials and Methods. These peptides were then analyzed. The peptide whose eluting position is indicated by the arrow was found to possess the following relative molar amino acid composition: Asp 0.109, Pro 0.154, Glu 0.100, Leu 0.103, Tyr 0.070, and Lys 0.212. This composition corresponds well with the residue 68–74 peptide. However, no radioactivity is seen to coelute with this peptide. Rather, three major peaks of radioactivity are seen to elute earlier. Because only a small proportion of the cytochrome *c* is actually methylated in the in vitro methylation, the resultant methylated peptide can be evidenced only by virtue of its radioactivity. These three radioactive peaks, designated as A, B, and C, were hydrolyzed and then analyzed. As shown in the lower part of Figure 4, peaks A, B, and C are seen to possess N^{ϵ}-trimethyl-, N^{ϵ}-dimethyl-, and N^{ϵ}-methyllysine, respectively. This result coupled with the fact that these peaks are in relatively close proximity to the unmethylated residue 68–74 peptide is indicative that they represent the methylated forms of this peptide with different degrees of methylation. It is somewhat surprising, however, that the methylation shifted the elution positions of the peptides to such an extent that they appear to overlap with other peptide peaks.

The wheat germ supernatant was used as a crude enzyme source to ascertain the presence or absence of additional cytochrome *c* methylase activities distinct from the residue 72 specific activity (of particular interest, a residue 86 methylating activity). The excellent substrate, denatured horse heart cytochrome *c*, was methylated with the supernatant and then was subjected to CNBr cleavage and subsequent Sephadex G-50 chromatography as described under Materials and Methods. This procedure has been previously shown to generate the following five CNBr fragments, which elute from Sephadex G-50 in the order as given (horse heart cytochrome *c* contains two methionine residues, residues 65 and 80): residue 1–80, 1–65, 66–104, 81–104, and 65–80 (Durban et al., 1978). The fact that residue 72 and 86 lysines exist on separate CNBr peptides (residue 65–80 and 81–104, respectively) enables this type of analysis to differentiate between these two potential sites of methylation.

Table IV: Effect of Various AdoHcy Analogues on Protein Methylase III Activity[a]

inhibitor added	% activity
none	100
S-inosyl-L-methionine	100
(hydroxyethyl)thioadenosine	100
n-butylthioadenosine	100
methylthioadenosine	100
S-adenosyl-D-homocysteine	67.4
S-adenosyl-L-homocysteine	27.4
S-adenosyl-DL-homocysteine	42.6

[a] 100% activity equals 9.79 pmol incorporated/min. 19.2 μg of enzyme protein and 1 mg of ethanol-denatured cytochrome *c* were used in the assays. All inhibitors were present at a final concentration of 40 μM.

Panel A of Figure 5 shows the Sephadex G-50 chromatogram for the CNBr-cleaved ethanol-denatured cytochrome *c*. For comparison, the purified yeast enzyme was used to methylate native horse heart cytochrome *c*, and that result is given in panel B. Because the yeast enzyme is known to have an exclusive residue 72 specificity, the two radioactive peaks labeled I and III in panel B can be assigned as the residue 66–104 and the residue 65–80 peptides. The residue 81–104 peak (II), which elutes between these two peaks, contains no radioactivity as would be expected (DiMaria et al., 1979). In panel A, it is clearly evident that the denatured cytochrome *c* methylated by the wheat germ enzyme possesses the same two radioactive peaks seen in the case of panel B with no radioactivity associated with the residue 81–104 peptide (peak II).

Inhibition by S-Adenosyl-L-homocysteine and Its Analogues. The availability of AdoHcy analogues prompted us to investigate the action of these compounds on the activity of the wheat germ enzyme. This type of study is the first to be performed for a protein (lysine) methyltransferase (protein methylase III). The other types of protein methylases thus far characterized, namely, protein methylases I and II, have already been studied in this regard (Casellas & Jeanteau, 1978; Enouf et al., 1979; Oliva et al., 1980). In Table IV, the results of the testing of various compounds for inhibitory activity are presented. Only the two forms of AdoHcy, D or L, either alone or in a racemic mixture were seen to be inhibitory. The other compounds as classified by modification of the adenine moiety, *S*-inosyl-L-homocysteine, or the homocysteine moiety, (hydroxyethyl)thioadenosine, *n*-butylthioadenosine, and methylthioadenosine, all showed no inhibitory activity whatsoever. Such a result indicates that the observed inhibition by the D or L forms of AdoHcy is strongly dependent on the integrity of both the adenine and homocysteine portions of the molecule.

In light of the work done with protein methylases I and II, several observations can be made. The inhibition of D-AdoHcy was also seen with protein methylase I (Casellas & Jeanteau, 1978; Enouf et al., 1979), but protein methylase II showed no such sensitivity toward D-AdoHcy (Oliva et al., 1980). It is also important to note that methylthioadenosine, although not inhibitory in this study, is a fairly strong inhibitor toward protein methylase II (Oliva et al., 1980).

Discussion

In an extension of the previous work done regarding cytochrome *c* methylation in fungi, it was of interest to consider

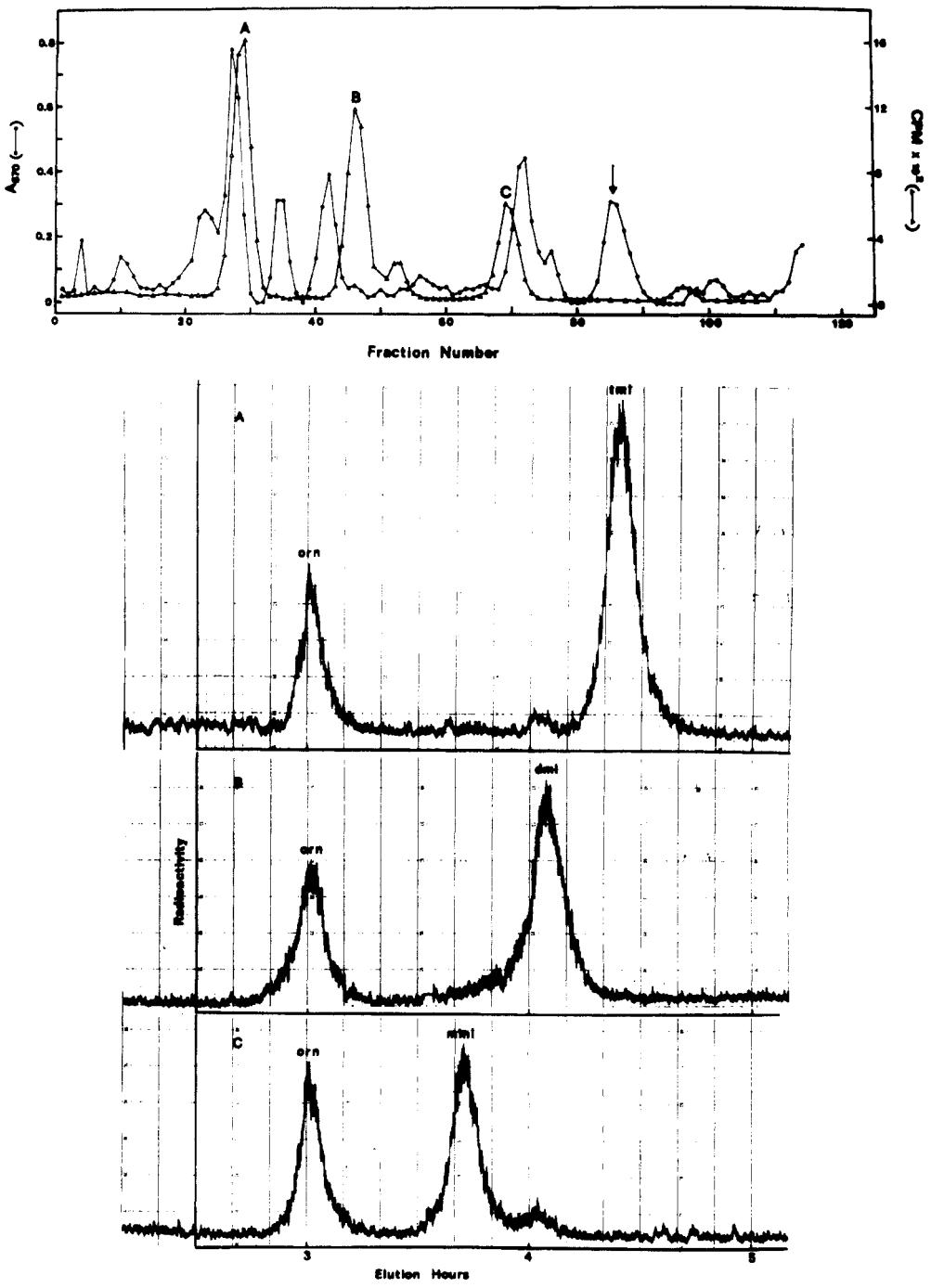

FIGURE 4: Chromatography of a chymotryptic digest of methylated [*methyl*-^{14}C]apocytochrome *c* on an Aminex A-5 column and subsequent analysis of the radioactive peaks. (Upper panel) 0.12 mg of apocytochrome *c* was in vitro methylated exactly as described in the Figure 4 legend with the wheat germ enzyme. After the incubation, 150 mg of horse heart apocytochrome *c* was added, and further preparation was done as described under Materials and Methods. The chymotryptic digest was applied to the Aminex A-5 column (1 × 60 cm) and eluted as described under Materials and Methods. Peptide elution was visualized by ninhydrin coloration (A_{570}) on the fractions. The eluting radioactivity profile shows the radiolabeled peptides. Peaks A, B, and C represent the major radiolabeled peptide peaks. The arrow indicates the ninhydrin positive peptide peak corresponding to the residue 72 containing residue 68–74 chymotryptic peptide. (Lower panel) Radioactive peptide peaks A, B, and C were further analyzed for N^{ϵ}-methylated lysines as described under Materials and Methods. The tracings represent radiograms of flow-cell monitoring of the eluting radioactivity from the amino acid analyzer. As an internal standard in these analyses, [^{14}C]ornithine was added to the hydrolysates.

this particular modification reaction in plant systems where an additional trimethyllysine exists at residue 86 in virtually all sequences thus far determined (Paik & Kim, 1980). This situation differentiates the two classes of organisms and suggests that the enzymology of the reaction in plants may differ from that which has been described for fungal systems.

In this study, we have sought to gain insight into this problem by purifying and studying cytochrome *c* methylase from wheat germ. One of our principal aims was to address the site(s) specificity of the DEAE-purified enzyme. In using native horse heart cytochrome *c* as a substrate, the enzyme was seen to possess an exclusive residue specificity that was identical with that of the yeast cytochrome *c* (residue 72 specific) methylase (see Figure 2). This observation was rather surprising in light of the multiple points of methylation that exist in wheat germ cytochrome *c*. It was further shown that while ethanol-denatured holocytochrome *c* and apocytochrome *c* were much better substrates than the corresponding native horse heart hemoprotein, this increase in substrate efficacy is not reflected in the recognition of new sites of methylation

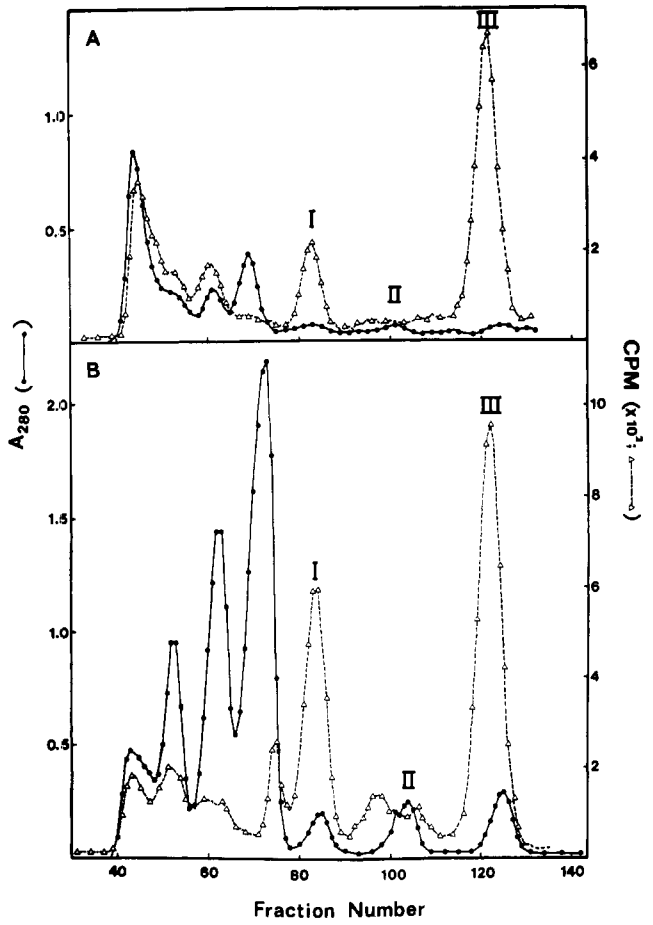

FIGURE 5: Column chromatographic analysis of CNBr peptides from ethanol-denatured horse heart cytochrome *c* methylated by crude wheat germ enzyme and comparison with native cytochrome *c* methylated by the purified yeast enzyme. (Panel A) 6 mg of denatured horse heart cytochrome *c* was methylated with 4.5 mg of crude wheat germ supernatant protein as a source of methylating enzyme in a final volume of 0.75 mL. After the incubation (40 min), the removal of unreacted AdoMet, CNBr cleavage, and subsequent chromatography on Sephadex G-50 (0.9 × 110 cm) were done as described under Materials and Methods. In the chromatographic profile, peaks I, II, and III denote the eluting positions of the CNBr peptides residue 66–104, 81–104, and 65–80, respectively. (Panel B) Native horse heart cytochrome *c* was methylated with purified yeast enzyme (DiMaria et al., 1979), and its peptides resulting from CNBr cleavage were analyzed as above. Peaks I, II, and III correspond to the same peptides listed above.

of which residue 86 would be a likely candidate. Rather an enhancement of the efficacy of methylation is localized to the residue 72 containing peptide (residue 68–74).

The failure to evidence an additional methylation site at residue 86 can be explained by a number of possibilities of which several will be considered here. For one, it can be envisioned that in order to be methylated, the cytochrome *c* substrate must possess certain stringent sequence requirements. This idea derives its credence from the fact that plant cytochromes *c* (e.g., wheat germ), while showing absolute homology in the residue 72 region (specifically residue 70–80) to animal and fungal cytochromes *c*, are significantly different when similarly compared in the region of the residue 86 (comparison of sequences given in Table V). In particular, it is seen that wheat germ possesses a proline at residue 88, contrasting sharply with the lysine or acidic amino acid seen in the corresponding position of animal and fungal cytochromes *c*, respectively. Thus, it is possible that while unmethylated plant cytochrome *c* is obviously enzymatically methylated at residue 86 in vivo, the enzyme responsible for this methylation

Table V: Cytochrome *c* Sequences in the Residue 72 and 86 Regions[a]

source of cytochrome *c*	residues	sequence[b]
wheat germ	84–90	-Gly-Leu-Lys-Lys-Pro-Gln-Asn-
horse heart	84–90	-Gly-Ile-Lys-Lys-Lys-Thr-Glu-
pigeon	84–90	-Gly-Ile-Lys-Lys-Lys-Ser-Glu-
yeast	84–90	-Gly-Leu-Lys-Lys-Glu-Lys-Asp-
Neurospora	84–90	-Gly-Leu-Lys-Lys-Asp-Lys-Asp-
all above species	70–80	-Asn-Pro-Lys[c]-Lys-Tyr-Ile-Pro-Gly-Thr-Lys-Met-

[a] Sequences taken from Dayhoff (1972). [b] Underlined residues are in vivo methylation sites. [c] Not methylated in horse heart or pigeon cytochromes *c*.

may not recognize horse heart or other nonplant cytochromes *c* due to differences in the amino acid sequence of this region of the molecule. This hypothesis, although attractive, was not able to be tested in this work due simply to the unavailability of unmethylated plant cytochrome *c*.

Another possibility that is likely is that an additional enzyme activity, responsible for residue 86 methylation, is lost in the progressive purification of cytochrome *c* methylating activity, leaving only the residue 72 specific activity. This possibility was examined by using a crude wheat germ supernatant as an enzyme source for the methylation of horse heart cytochrome *c*. As was reported in Figure 5, the results of this approach failed to reveal any methylation associated with the residue 86 containing CNBr peptide, residue 86–104 (peak II). This apparent lack of residue 86 specific activity is also inferred from the fact that the crude wheat germ supernatant failed to methylate the various forms of yeast cytochrome *c*. These cytochromes *c* can potentially be methylated at residue 86 but not at residue 72 (Table III).

Other reasons for the lack of this activity can be advanced, but in light of the fact that cytochrome *c* methylation is shown to occur virtually concomitant with peptide backbone synthesis (perhaps when the nascent polypeptide is polysome bound) (Farooqui et al., 1980), it is particularly tempting to speculate that the nascent cytochrome *c* as it exists in this polysome-bound complex may be the obligatory substrate for residue 86 methylation. This proposal derives credence from the fact that the in vivo methylation of myosin is actually seen to occur cotranslationally (Reporter, 1973).

The residue 72 specific activity was found to possess several characteristics that were not observed with the fungal enzymes (Durban et al., 1978; DiMaria et al., 1979). For one, the wheat germ enzyme was able to sharply differentiate between cytochromes *c* of various species with respect to substrate preference. In this regard, the avian cytochromes *c*, pigeon and chicken, were much better substrates than those of mammalian origin. This observation may reflect the possibility that the enzyme is able to recognize subtle differences in protein tertiary structure since the amino acid differences between avian and mammalian species of cytochrome *c* are quite minimal; for example, horse and chicken cytochromes *c* differ in only 11 out of 104 amino acid residues (Dayhoff, 1972). In addition, none of these differences are in close proximity to the residue 72 methylation site.

Another finding was the extremely low K_m that was obtained for apocytochrome *c*. The value (1.12 μM) is much lower than that observed for the methylases of fungal origin (Durban et al., 1978; DiMaria et al., 1979). It is interesting to speculate that this relatively low K_m may reflect the fact that the concentration of cytochrome *c* in wheat germ, like most plants, is extremely low when compared with that of *Neurospora* or

yeast (Hagihara et al., 1959). In addition, the excellent substrate capability of this protein is also consistent with the proposal that methylation of cytochrome *c* occurs before heme attachment, perhaps as suggested earlier when the protein is polysome bound (Farooqui et al., 1980).

As a corollary to this work, the anomalous behavior of the methyl-, dimethyl-, and trimethyllysine forms of the residue 68–74 chymotryptic peptide in relation to their unmethylated counterpart on Aminex A-5 chromatography should be discussed. The apparent decrease in affinity of the peptide to the Aminex resin, which appears to be a function of the degree of methylation, can be interpreted as a decrease in the basicity of the peptide. It is tempting to correlate this behavior with that of methylated and unmethylated cytochrome *c* on cation-exchange resins as well as isoelectric-focusing columns. In these cases, the methylated form of the holoprotein is seen to be less basic than the unmethylated form (Scott & Mitchell, 1969; Kim et al., 1980). Thus, from the present observations, it is possible that the effect of methylation at the holoprotein level may actually reflect a localized alteration around the methylated lysine residue rather than a generalized change in protein conformation as has been proposed (Kim et al., 1980).

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