Site Specificity of Histone H4 Methylation by Wheat Germ Protein-Arginine *N*-Methyltransferase†

Suhas G. Disa, Arun Gupta,‡ Sangduk Kim, and Woon Ki Paik*
Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania 19140
Received September 30, 1985; Revised Manuscript Received December 18, 1985

ABSTRACT: CNBr treatment of calf thymus [*methyl*-^{14}C]histone H4, methylated in vitro with *S*-adenosyl-L-[*methyl*-^{14}C]methionine by a highly histone-specific wheat germ protein methylase I (*S*-adenosyl-L-methionine:protein-L-arginine *N*-methyltransferase, EC 2.1.1.23), produced two peptide fragments corresponding to residues 1–83 and 84–102, with the former being radioactive. Two-dimensional peptide mapping of the chymotryptic and tryptic digest of [*methyl*-^{14}C]histone H4 and analysis of the chymotryptic digest on HPLC have shown that only a single peptide is radiolabeled. In order to define the exact site of methylation (arginine residue), the radioactive peptide from the chymotryptic digest of [*methyl*-^{14}C]histone H4 was further purified on HPLC by linear and then isocratic elution. The purified chymotryptic peptide was then digested with trypsin and purified on HPLC, and its amino acid composition was determined on HPLC. These results indicate that the peptide corresponding to residues 24–35 of histone H4 is radiolabeled. Since this peptide contains a single arginine residue at position 35, we have concluded that the enzyme is specific not only to the protein substrate but also to the methylation site.

Recently, we have identified and partially purified a highly histone-specific protein methylase I (*S*-adenosyl-L-methionine:protein-L-arginine *N*-methyltransferase, EC 2.1.1.23) from wheat germ (Gupta et al., 1982). Even such proteins as myelin basic protein (AI basic protein or MBP) and HMG-1 and HMG-2 proteins (high-mobility-group proteins), which are shown to be in vivo methylated (Paik & Kim, 1980), were completely inactive as substrates for the enzyme in vitro. When the partially purified protein methylase I was reacted with wheat germ extract in the presence of *S*-adenosyl-L-[*methyl*-^{14}C]methionine, only endogenous histones H4 and H2B were methylated. However, earlier studies on the amino acid sequence of various histones failed to detect the presence of N^G-methylarginine (DeLange et al., 1969a; Ogawa et al., 1969). Since wheat germ protein methylase I is highly specific toward histone H4 and this histone contains 14 arginine residues, the present study was undertaken to determine whether this enzyme indiscriminately methylates arginine residues or specifically methylates certain arginine residue(s) requiring a highly specialized sequence for methylation.

Materials and Methods

Materials. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine (specific activity 60 mCi/mmol) was obtained from Amersham Nuclear Corp. Cyanogen bromide, TLCK- (N^{α}-*p*-tosyllysine chloromethyl ketone) treated α-chymotrypsin (trypsin activity inhibited, type VII), and Sephadex G-50 were purchased from Sigma Chemical Co. TPCK- [1-(tosylamido)-2-phenylethyl chloromethyl ketone] treated trypsin (chymotrypsin activity inhibited) was obtained from Worthington Biochemicals. Highly purified calf thymus histone H4 was from Boehringer Mannheim Biochemicals. HPLC[1]-grade acetonitrile was purchased from Tedia Biochemical Co. and trifluoroacetic acid from J. T. Baker and Co. The HPLC system, including various columns used, was from Waters Associates. Partially purified protein methylase I was prepared from wheat germ according to the method described earlier (Gupta et al., 1982). Histones from wheat germ were isolated by the method of Fazal and Cole (1977). All other reagents used were obtained commercially and were of the highest purity available.

Methylation of Histone H4. Methylation of histones was carried out by the procedure previously described (Gupta et al., 1982). Briefly stated, in a final volume of 2.8 mL, the assay mixture contained 0.4 mL of 1 M glycine–NaOH buffer, pH 9.0, 0.4 mL of 0.1 M dithiothreitol in 30% glycerol and 0.1 M glycine–NaOH buffer, pH 9.0, 0.4 mL of *S*-adenosyl-L-[*methyl*-^{14}C]methionine (100 nmol, 90–100 cpm/pmol), and 0.4 mL of histone substate (12 mg of pure histone H4). After incubation at 37 °C for 2 h, the reaction was terminated by the addition of 3.0 mL of 15% trichloroacetic acid (TCA). Phospholipids, nucleic acids, and protein carboxymethyl esters produced by contaminating protein methylase II (*S*-adenosyl-L-methionine:protein-L-glutamate *O*-methyltransferase, EC 2.1.1.24) were removed as described (Kim & Paik, 1978). Finally, the methylated histone was washed twice with absolute alcohol and hydrolyzed with chymotrypsin, trypsin, or CNBr.

CNBr Treatment and Separation of Peptides. The enzymatically methylated [*methyl*-^{14}C]histone H4 was cleaved with CNBr by the method described (DeLange et al., 1968). Ten milligrams of [*methyl*-^{14}C]histone H4 was dissolved in 70% formic acid with an 80-fold molar excess of CNBr in a final volume of 2.0 mL. The reaction was then carried out in the dark at room temperature on a rotary shaker for 20 h. At the end of the reaction, the mixture was diluted with 20 mL of deionized water and lyophilized to dryness over NaOH pellets. The lyophilized sample was dissolved in 0.2 mL of 90% formic acid and further diluted to 1.0 mL with water. This sample was loaded on a Sephadex G-50 column (0.9 cm × 155 cm) that had been previously equilibrated with 30% acetic acid.

† Supported by Research Grants AM09602 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, CA12226 and CA12227 from the National Cancer Institute, and RG1765-A-1 from the National Multiple Sclerosis Society.
* Author to whom correspondence should be addressed.
‡ Present address: Department of Biological Chemistry, University of Cincinnati College of Medicine, Cincinnati, OH 45267.

[1] Abbreviations: HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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The column was eluted with 30% acetic acid, and 2.0-mL fractions were collected. The peptide was detected by hydrolyzing 0.2 mL of each fraction with 1.0 mL of 2.5 N NaOH at 90 °C for 2.5 h and then reacting with ninhydrin by the method of Hirs et al. (1956). Aliquots of 0.2 mL from the same fractions were also taken for radioactivity determination.

Chymotryptic Digestion of [methyl-^{14}C]Histone H4 and Peptide Mapping. [*methyl*-^{14}C]Histone H4 was subjected to chymotryptic digestion as described by Brautigan et al. (1978). Specifically, 100 mg of enzymatically methylated [*methyl*-^{14}C]histone H4 was dissolved in 10.0 mL of 0.1 M ammonium bicarbonate, pH 8.0. Ten milligrams of TLCK-treated α-chymotrypsin (tryptic activity inhibited) was added to the above protein suspension, and the digestion was carried out for 20 h at 28 °C with intermittent shaking. At the end of the incubation, the reaction mixture was lyophilized. Lyophilization was repeated an additional 2 times to completely remove ammonium bicarbonate. The lyophilized sample was dissolved in a minimum amount of electrophoretic buffer (water:pyridine:acetic acid = 1800:100:4 v/v) and spotted on Whatman 3MM paper for peptide mapping. The running conditions for the two-dimensional peptide mapping procedure were the same as described by DiMaria et al. (1979). The peptides were detected with 0.1% ninhydrin in ethanol. Autofluorography of the map was done by using Kodak X-ray-sensitive film with an exposure time of 4–5 days.

Tryptic Digestion of [methyl-^{14}C]Histone H4. Tryptic digestion of enzymatically labeled [*methyl*-^{14}C]histone H4 was performed as described by DeLange et al. (1969b). Briefly, 50 μg of TPCK-treated trypsin (chymotryptic activity inhibited) was added to 10 mg of [*methyl*-^{14}C]histone H4 dissolved in 0.1 M ammonium bicarbonate, pH 8.0. The digestion was allowed to proceed at 40 °C for 6 h, and the reaction was stopped by the addition of glacial acetic acid. The sample was lyophilized 3 times in order to remove ammonium bicarbonate. The two-dimensional peptide mapping was carried out in a similar way as described above.

Purification of Radioactive Peptide from Chymotryptic Digest. For further characterization of the radioactive peptide, the chymotryptic digest of enzymatically labeled [*methyl*-^{14}C]histone H4 was further purified by HPLC as described by Sasagawa et al. (1982). The chymotrypsin-digested sample was dissolved in 0.1% TFA in water, and 100 μL was injected into an HPLC μBondapak C_{18} column (Waters Associates) that had been equilibrated with 0.1% TFA in water. Various peptides were then eluted with a linear gradient of acetonitrile (0–60% acetonitrile in water) in a 60-min run with a 1% gradient slope per minute at a flow rate of 2.0 mL/min. Two-milliliter fractions were collected, and the elution was monitored at 220 nm. The radioactive peptide was detected by measuring the radioactivity of 50-μL aliquots from the fractions in a Packard Tri-Carb scintillation counter. The radioactive fractions were pooled and frozen. The frozen sample was then lyophilized and washed 4 times with water. This lyophilized sample was dissolved in a minimum amount of 0.1% TFA in 5% acetonitrile.

In order to remove any contaminating peptides, the sample was then again injected into an HPLC column and eluted isocratically with 0.1% TFA in 5% acetonitrile at a flow rate of 1.0 mL/min. The elution was monitored at 220 nm, 1.0-mL fractions were collected, and radioactivity was determined from the sample aliquot. The radioactive fractions were pooled, frozen, and lyophilized. The lyophilized sample was washed 3 times with water, again dissolved in 0.1% TFA in 5% acetonitrile, and reinjected into the HPLC column to further purify as well as to ascertain the homogeneity of the isolated peptide. The peptide was eluted as described above. The peptide peak containing radioactivity was collected in its entirety, frozen, and lyophilized for further digestion with trypsin (see below).

Digestion of Purified Chymotryptic Methyl-^{14}C-Labeled Peptide with Trypsin. The lyophilized sample obtained above was dissolved in 0.5 mL of 0.2 N *N*-ethylmorpholine acetate, pH 8.1, and 50 μg of TPCK-treated trypsin was added. The digestion was carried out at 37 °C for 24 h as described by Fullmere and Wasserman (1979). The reaction was stopped by the addition of glacial acetic acid, and the sample was injected into the HPLC column without further manipulation.

Purification of Trypsin-Digested Methyl-^{14}C-Labeled Peptide on HPLC. In order to purify the methyl-^{14}C-labeled peptide, the above trypsin-digested sample was injected directly into the HPLC system. The elution of the peptides was carried out isocratically with 0.1% TFA in 5% acetonitrile with a flow rate of 1.0 mL/min. The peptide peak was monitored at 220 nm. Simultaneously, aliquots from various peaks were monitored for radioactivity. The peak fractions containing radioactivity were collected, frozen, and lyophilized. This lyophilized sample was washed thoroughly (at least 4 times) with water before hydrolyzing for amino acid composition analysis.

Amino Acid Analysis of Purified Methyl-^{14}C-Labeled Peptide. The lyophilized tryptic peptide isolated above on HPLC was dissolved in 0.50 mL of 20% formic acid–2 N HCl and quantitatively transferred into a Pierce digestion tube. The tube was then depressurized and hydrolysis carried out at 110 °C for 4 h on a heating block. At the end of the digestion period, the tube was allowed to cool to room temperature, frozen, and dried in vacuo. This dried sample was then washed thoroughly with water (4 times) and dissolved in buffer A (methanol:tetrahydrofuran:water = 2:2:96 with 50 mM sodium acetate and 50 mM sodium phosphate, dibasic, pH 7.5). The amino acid analysis was performed on HPLC as described earlier (Suh et al., 1986), using method 2 analysis (*Amino Acid Analysis System Operator's Manual*, 1984).

RESULTS

Sephadex G-50 Column Chromatography of CNBr-Treated Methyl-^{14}C-Labeled Calf Thymus Histone H4. Calf thymus histone H4 has one methionine residue at position 84 (DeLange et al., 1968). Thus, CNBr treatment of histone H4 is expected to give rise to two peptides, one representing residues 1–83 and another residues 84–102. The elution profile of CNBr-treated histone H4 from the Sephadex G-50 column is shown in Figure 1. As expected, we could detect two well-separated ninhydrin-positive peaks. However, the radioactivity was found to be present in only one peak, which was eluted earlier than the other (thus, on the larger peptide). This result suggests that the methylation site may reside at the N-terminal fragment of residues 1–83 of histone H4.

Peptide Mapping of [methyl-^{14}C]Histone H4. Calf thymus histone H4 contains 14 arginine residues (DeLange et al., 1968). Thus, the question arises as to which arginine residue(s) has (have) been methylated and with what specificity. In order to probe further into this question, enzymatically methylated [*methyl*-^{14}C]histone H4 was digested with either α-chymotrypsin or trypsin, and the respective digests were subjected to two-dimensional peptide mapping as described under Materials and Methods. The peptide maps of chymotryptic and tryptic digests are illustrated in panels A and C and panels B and D of Figure 2, respectively. The peptides visualized by ninhydrin coloration are outlined, and those that are radioactive are shaded. It is evident from these results that out of

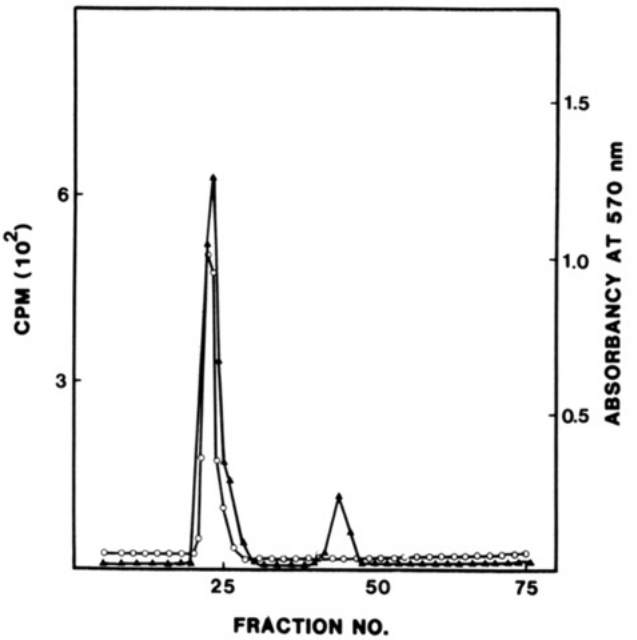

FIGURE 1: Sephadex G-50 column chromatography of CNBr peptide of enzymatically methylated [*methyl*-^{14}C]histone H4. Fractions of 2 mL were collected at a flow rate of 20 mL/h from the Sephadex G-50 column (0.9 cm × 155 cm) equilibrated and eluted with 30% acetic acid at room temperature. Eluted peptides were detected by ninhydrin reaction after alkaline hydrolysis, and radioactivity was monitored directly by drawing aliquots and mixing with scintillation fluid. (○) Radioactivity; (▲) A_{570}. The rest of the experimental procedure is described under Materials and Methods.

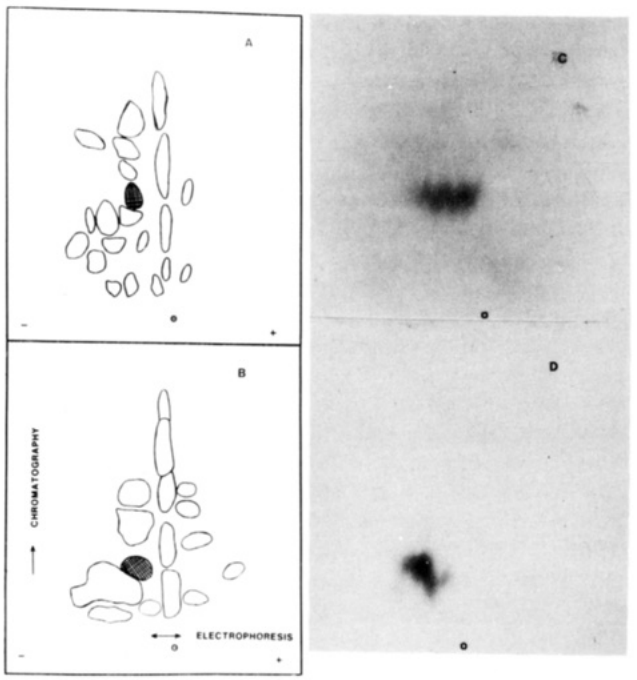

FIGURE 2: Two-dimensional peptide mapping of chymotryptic (A, C) and tryptic (B, D) digest of enzymatically methylated [*methyl*-^{14}C]histone H4. A small circle indicates the application point of the sample. Electrophoresis in the horizontal dimension and chromatography in the vertical dimension were performed sequentially. Outlined areas indicate ninhydrin-positive spots and hatched areas indicate radioactive spots, as visualized by autofluorography (panels C and D). Panel C represents an autofluorograph of the chymotryptic digest (panel A) and panel D for the tryptic digest (panel B), respectively. A more detailed description of the procedure is given under Materials and Methods.

the many ninhydrin-positive spots only one is radioactive in both maps, as demonstrated by autofluorography. The radioactive spots moved considerably in the chromatographic direction and in the cathodic direction (negative electrode) in electrophoresis.

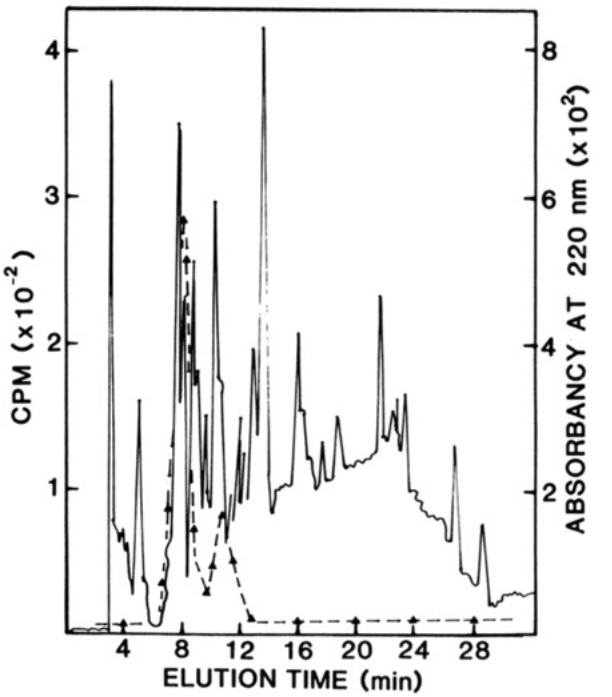

FIGURE 3: High-performance liquid chromatography of chymotryptic-digested [*methyl*-^{14}C]histone H4. The digest was dissolved in initial buffer, and 0.100 mL was injected into the HPLC system. The peptides were then eluted with a linear gradient of acetonitrile from 0.1% TFA in water to 60% acetonitrile in 0.1% TFA in water in 60 min with a gradient slope of 1% per minute at a flow rate of 2.0 mL/min. Two-milliliter fractions were collected. Peptide peaks were monitored at 220 nm. Fifty-microliter aliquots were removed from each fraction and mixed with scintillation cocktail, and radioactivity was determined. (—) A_{220}; (▲) radioactivity.

Chymotrypsin and trypsin clearly have different cleavage sites. However, in both the digestions we could detect a single radioactive spot in the entire map, which strongly indicates that the wheat germ protein methylase I is highly specific to the site of arginine methylation and may not methylate arginine residues at random.

Further Purification and Characterization of Radioactive Peptide on HPLC. In order to characterize the radioactive peptide from the chymotryptic digest of enzymatically labeled [*methyl*-^{14}C]histone H4, we employed an HPLC system for further purification of this peptide. When the chymotryptic digest was separated on HPLC using a linear gradient of acetonitrile and elution was monitored at 220 nm, many peptides were separated in a 60-min run (Figure 3). As can be seen, the major radioactivity was associated with only one peptide. A minor radioactive peak was also eluted after the major one, having about 25% of the total radioactivity. This peak could either be due to an incomplete digestion of histone H4, another methylation site, or, most likely, an N^G,N'^G-dimethylarginine-containing species (as described in Figure 7, the major radioactivity was associated with N^G-methylarginine).[2] Because of very low yield, further studies on this minor peak were not carried out.

It has been shown by Fullmere and Wasserman (1979) that peptide isolated on HPLC in a linear gradient could be further resolved in an isocratic elution system, which otherwise is not

[2] It has previously been observed that the acid hydrolysate of whole histone H4 methyl-^{14}C-labeled by wheat germ protein methylase I yielded both N^G-[^{14}C]methyl- and N^G,N^G-di[$^{14}C_2$]methylarginines (Gupta et al., 1982).

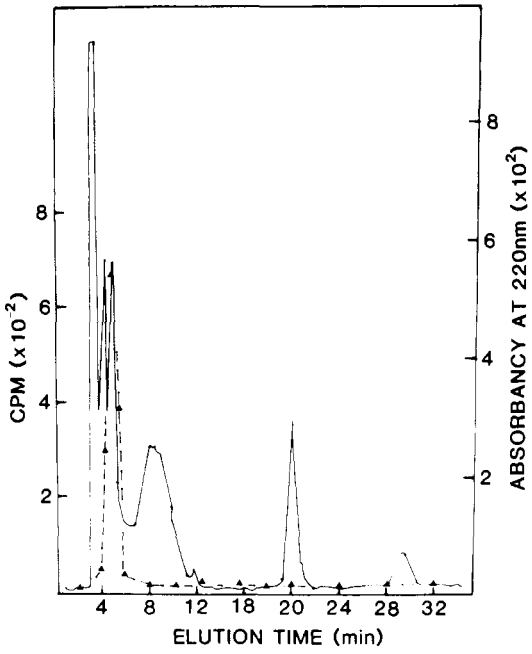

FIGURE 4: Further purification of radioactive peptide isolated on linear gradient (Figure 3) with isocratic elution on HPLC. The radioactive peptide preparation eluted from HPLC in a linear gradient of acetonitrile (Figure 3) was dissolved in 0.1% TFA with 5% acetonitrile. The 100-μL sample was injected into the HPLC and eluted isocratically with 0.1% TFA in 5% acetonitrile in a 35-min run at a flow rate of 1.0 mL/min. One-milliliter sample fractions were collected. Fifty-microliter aliquots were removed for radioactivity determination. The elution was monitored at 220 nm. (—) A_{220}; (▲) radioactivity.

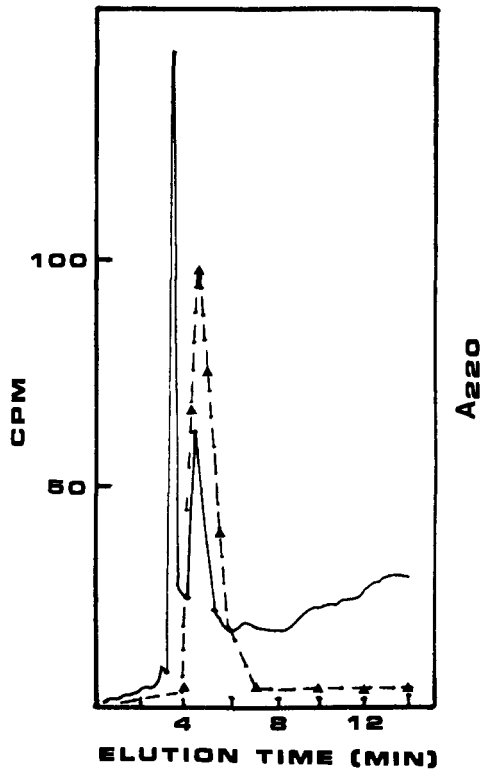

FIGURE 5: Rechromatography of isocratically eluted radioactive peptide (Figure 4) on HPLC. All the experimental conditions were as given under Figure 4 and Materials and Methods.

separated. When we adapted the isocratic elution system, the peptide separated in a linear gradient was further resolved (Figure 4). The radioactivity again eluted entirely in one of the peptide peaks. When this peptide was further rechromatographed in the same system, it showed a single homogeneous peak containing all the radioactivity (Figure 5).

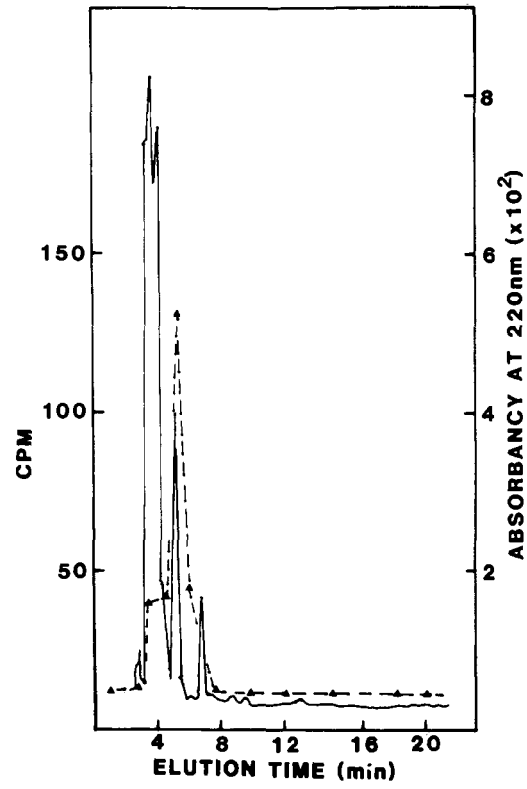

FIGURE 6: HPLC separation of tryptic-digested radioactive peptide. The radioactive peptide separated in the previous experiment (Figure 5) was further digested with TPCK-treated trypsin as given under Materials and Methods and injected directly into the HPLC system. Various peptides were eluted from the column with 0.1% TFA in 5% acetonitrile in a 20-min run at a flow rate of 1.0 mL/min. One-milliliter fractions were collected, and radioactivity was measured on 50-μL aliquots from each fraction. The elution profile was monitored at 220 nm. (—) A_{220}; (▲) radioactivity.

In order to ascertain which arginine residue(s) had been methylated, the radioactive peptide isolated from the chymotryptic digest and purified by HPLC, as described above, was further treated with trypsin. The trypsin digest of this radioactive peptide was again subjected to HPLC separation. As shown in Figure 6, among a few peaks, the peak eluting at 5.16 min contained more than 90% of the radioactivity loaded.

Amino Acid Analysis and Site of Methylation. In order to identify the site of the arginine residue methylated, the above-mentioned radioactive peptide isolated from the chymotryptic and tryptic digest was hydrolyzed and subjected to amino acid analysis on HPLC, using *o*-phthaldialdehyde as a fluorescent labeling reagent. As shown in Figure 7, the radioactivity was eluted with a peak emerging at 16.75 min of retention time. This peak was identified in a separate chromatographic experiment as N^G-methylarginine (result not shown). Table I shows the composition of this peptide. A minimum of 11 amino acids were detected. [*o*-Phthaldialdehyde used for HPLC analysis is specific toward primary amines, but not secondary. Thus, proline is not reactive with this reagent (Roth, 1971).] When this composition was compared with the already published sequence data (DeLange et al., 1969a; Ogawa et al., 1969), it corresponded very closely to the sequence of residues 24–35, except that we could not detect any threonine but instead found serine.

Discussion

In our present studies, calf thymus histone H4, when methylated in vitro with *S*-adenosyl-L-[*methyl*-^{14}C]methionine

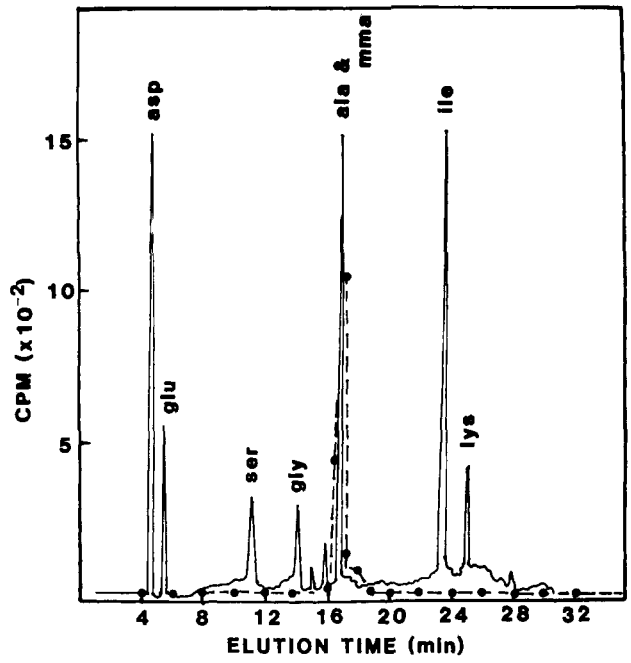

FIGURE 7: Amino acid analysis of radioactive tryptic-digested peptide (Figure 6) on HPLC. The radioactive peptide purified in the previous step (Figure 6) was hydrolyzed for amino acid analysis as described under Materials and Methods. The 10-μL digest was injected into the HPLC system, and analysis was carried out as described elsewhere (Suh et al., 1986). (—) Amino acid peak indicated by fluorescence (excitation, 334 nm; emission, 425 nm); (●) radioactivity. mma represents N^G-methylarginine.

Table I. Amino Acid Composition of Methyl-^{14}C-Labeled Peptide Isolated from Calf Thymus [*methyl*-^{14}C]Histone H4

amino acid detected	obsd amt (pmol/μL of sample)	min no. of residues calcd	no. of residues assumed
aspartic acid + asparagine	11.29	1.83	2
glutamic acid + glutamine	5.13	0.83	1
serine	6.47	1.05	1
glycine	4.87	0.79	1
alanine + N^G-methylarginine[a]	14.99	2.43	2
isoleucine	17.42	2.83	3
lysine	6.16	1.00	1

[a] These amino acids coelute on HPLC. The amino acid sequence of residues 24–35 of calf thymus histone H4 is Asp-Asn-Ile-Gln-Gly-Ile-Thr-Lys-Pro-Ala-Ile-Arg (DeLange et al., 1969a). It should be noted that proline is not reactive with *o*-phthaldialdehyde (Roth, 1971).

by wheat germ histone-specific protein methylase I, seems to be primarily methylated in one peptide, which resulted from the chymotryptic and tryptic digest of this protein. This methyl-^{14}C-labeled peptide, which was purified to homogeneity through repeated runs on HPLC under various conditions (Figures 4–6), has a minimum of 11 amino acids, exclusive of proline. This composition matched very well with residues 24–35 having the following sequence: Asp_{24}-Asn-Ile-Gln-Gly-Ile-Thr-Lys-Pro-Ala-Ile-Arg_{35} (DeLange et al., 1969a; Ogawa et al., 1969) (Table I). These results undoubtedly suggest that the arginine residue at position 35 has been methylated. This arginine residue was found to be monomethylated, as judged by the retention time of this amino acid derivative on amino acid analysis by HPLC [this work and Gupta et al. (1982)].

A closer look at this peptide reveals that it has a Lys–Pro bond that was not cleaved with tryptic digestion. There are a few reports indicating anomalies of tryptic action on protein or peptide fragments (Maroux et al., 1966; Plapp et al., 1967; Bachmayer et al., 1968). In their detailed studies on specificity of trypsin action, Sanborn and Hein (1968) have suggested that trypsin specificity is highly dependent upon the length of the side chain containing a positive charge and its nature and orientation with respect to the positive charge. Furthermore, DeLange et al. (1969a) has pointed out that the Lys–Pro bond in histone H4 is generally resistant to tryptic attack. In light of these observations, it is not surprising that the Lys–Pro bond in our peptide preparation was not cleaved by trypsin, and in all probability, therefore, the arginine residue at position 35 is the site of enzymatic methylation. If this is the case, then the absence of the threonine peak and the presence of the serine peak in the chromatogram (Figure 7) seem unexpected. One possibility could be that threonine, being a labile amino acid, could have been destroyed during hydrolysis. Alternatively, serine and threonine are very similar amino acids, and threonine may have been present but was not detected by earlier workers.

Histones are probably the most conserved of all proteins studied so far. Histone H4 seems to be extremely well conserved from species as removed as pea and calf (DeLange et al., 1969b). In the N-terminal portion of this histone molecule, there are two clusters of basic amino acids, the first from residues 16–20 and the second from residues 34–40. In the first cluster, amino acids 16 and 20 are acetylated and methylated, respectively, and these have been implicated in the interaction of histone H4 with DNA (Van Holde & Isenberg, 1975). Thus, it seems quite likely that, as in the first cluster of basic amino acids, the second cluster may have a modification site and, being close to the first one, may further help to participate in the interaction with DNA. Such site of modification then could be at position 35. However, it should be noted that the observations reported herein were carried out with calf thymus histone H4 by a wheat germ protein methylase I in vitro. There is no evidence of this type of reaction occurring in vivo.

Registry No. Arg, 74-79-3; protein-arginine *N*-methyltransferase, 9055-07-6.

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Studies of Thermally Induced Denaturation of Azurin and Azurin Derivatives by Differential Scanning Calorimetry: Evidence for Copper Selectivity†

Helen R. Engeseth and David R. McMillin*
Department of Chemistry, Purdue University, West Lafayette, Indiana 47907
Received August 20, 1985; Revised Manuscript Received December 6, 1985

ABSTRACT: Azurin, a blue copper protein from *Pseudomonas aeruginosa*, and several derivatives of azurin have been studied by differential scanning calorimetry. Two well-separated, irreversible transitions are observed in a scan of apoazurin under a variety of conditions, and they are assigned to distinct steps in the denaturation process. No specific structural component can be assigned to the lower temperature transition, but a "flap" structure which is found near the metal binding site may be involved. Circular dichroic spectra suggest that melting of the β-sheet structure, the main structural motif in the native protein, occurs during the second transition. With the exceptions of the Ni(II) and *p*-(hydroxymercuri)benzoate derivatives, the transitions are superposed in the metalated forms, and the enthalpies of denaturation are more endothermic. By comparison with other first-row divalent transition ions and especially Zn(II), the Cu(II) derivative exhibits the most endothermic denaturation process. Along with other data, this suggests that the binding energy is greater for Cu(II). It is postulated that the selectivity for copper over zinc arises because of the irregular binding geometry offered by the folded protein. Denaturation of the Hg(II) derivative is even more endothermic, confirming that the type 1 binding site has a very great affinity for Hg(II). Finally, when substoichiometric amounts of Hg(II) are added to the apoprotein, there is evidence that a novel mercury-bridged dimer of azurin forms.

The type 1 or "blue" copper center occurs in a number of proteins and has quite distinctive spectroscopic properties. Its hallmark is an intense visible absorbance in the region of 600 nm where the molar absorptivity is typically ca. 5000 M^{-1} cm^{-1}. A second characteristic signature is an unusually small hyperfine splitting from the copper nucleus in the so-called parallel region of the electron paramagnetic resonance spectrum (Fee, 1975; Gray & Solomon, 1981). The small blue copper proteins like plastocyanin, which is found in chloroplasts, and azurin, which is a bacterial protein, are believed to function as electron transferases (Farver & Pecht, 1984; Adman, 1985). These proteins tend to occur at the high-potential end of electron-transport systems; accordingly, the reduction potential of a blue copper center is fairly positive and usually greater than that of the aqueous Cu(II)/Cu(I) couple (Gray & Solomon, 1981).

The spectroscopic properties can be rationalized in terms of structure, now that crystal and molecular structures are available for the oxidized forms of plastocyanin from *Populus nigra* var. *italica* (Gus & Freeman, 1983) and the azurins from *Pseudomonas aeruginosa* (Adman & Jensen, 1981) and *Alcaligenes denitrificans* (Norris et al., 1983). The plastocyanin structure is known in most detail, and it reveals that there are four ligands disposed in a distorted tetrahedral fashion about copper. The donor set is comprised of two nitrogens from imidazole groups and a cysteine sulfur, each 2.0–2.1 Å removed from Cu(II), and a methionine sulfur which is found significantly farther away (ca. 2.9 Å). In the azurins, the copper environment is similar except that a fifth donor, a peptide oxygen, may be close enough to interact with the copper. The presence of the cysteine sulfur gives rise to a low-energy sulfur to copper charge-transfer absorption which is primarily responsible for the intense visible absorbance (Gray & Solomon, 1981; McMillin et al., 1974; McMillin & Morris, 1981). The tetrahedral coordination geometry along with covalent interactions involving the thiolate ligand explains the narrow hyperfine splitting (Brill, 1977; Malmström & Vänngård, 1960; Bencini & Gatteschi, 1983; Penfield et al., 1985).

The protein structure also influences the redox reactivity of the copper center. The prevailing view is that by presenting a rather rigid, distorted tetrahedral binding site, the protein conformation provides for a relatively positive reduction potential and a small Franck–Condon barrier for the electron-transfer reaction (Gray & Solomon, 1981; Farver & Pecht, 1981, 1984; Holwerda et al., 1976; Gray & Malmström, 1983). Because Cu(II) is generally found in a tetragonal environment with a coordination number of 5 or 6, the blue copper site appears to be biased toward the binding of Cu(I). In addition,

† This research was supported by Grant GM 22764 from the National Institutes of Health.

0006-2960/86/0425-2448$01.50/0