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Effect of enzymic methylation of heterogeneous ribonucleoprotein particle A1 on its nucleic-acid binding and controlled proteolysis

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Recombinant unmethylated heterogeneous nuclear ribonucleoprotein particle (hnRNP) protein A1 was enzymically methylated by nuclear protein/histone protein methylase I [Rajpurohit, Lee, Park, Paik and Kim (1994) J. Biol. Chem. **269**, 1075–1082] and the effect of methylation on several physicochemical properties was studied. The relative binding-affinity of methylated and unmethylated protein A1 to nucleic acid was quite different. This was observed by the elution behaviour of the protein A1 on a single-stranded DNA/cellulose column; the concentration of NaCl required to release the bound protein A1 was 0.59 M for the methylated and 0.63 M for the unmethylated, respectively. Employing isoelectrofocusing, pI values of the methylated and unmethylated proteins were found to be 9.41 and 9.48, respectively. Maximum fluorescence quenching of protein A1 in the presence of coliphage MS2-RNA was found to be 40 % with methylated and 45 % with unmethylated. When both species of protein A1 were subjected to controlled trypsin digestion, $t_{\frac{1}{2}}$ of the methylated protein was 1.31 min and the unmethylated, 1.63 min. The difference in their $t_{\frac{1}{2}}$ values was much greater in the presence of MS2-RNA; 2.4 min for the former and 4.3 min for the latter, indicating that the methylated species was less stabilized by the RNA than the unmethylated. All of the above results consistently suggested that the binding-property of hnRNP protein A1 to single-stranded nucleic acid was significantly reduced subsequent to its arginine-methylation. The biological significance of this observation is discussed.

INTRODUCTION

Shortly after biosynthesis, heterogeneous RNAs in eukaryotes combine with a set of proteins to form an RNA–protein complex particle known as the heterogeneous ribonucleoprotein particle (hnRNP). This structure is known to serve as the platform to form mRNA after a series of reactions involving splicing, packaging and transport to cytoplasm (Dreyfuss, 1986; Choi et al., 1986; Sierakowska et al., 1986). Several structurally related proteins (A1, A2, B1, B2, C1 and C2) are associated with the particle, protein A1 being the major core protein (Beyer et al., 1977; Karn et al., 1977). Protein A1 is a basic protein with molecular mass of 34 kDa, consisting of 320 amino acid residues (Herrick and Alberts, 1976; Kumar et al., 1986; Cobianchi et al., 1986) Characteristics of protein A1 are to bind single-stranded (ss) RNA and ssDNA, to stimulate α DNA polymerase *in vitro* (Herrick et al., 1976; Riva et al., 1986), and to contain post-translationally formed N^G-dimethylarginine residues (Karn et al., 1977; Kumar et al., 1986).

The protein A1 contains two distinct domains: N-terminal (residues 1–196) and glycine-rich C-terminal regions (residues 197–320) (Kumar et al., 1986; Cobianchi et al., 1986). These domain fragments can easily be generated *in vitro* by limited trypsin digestion (Kumar et al., 1990) or *in vivo* by endogenous protease (Herrick and Alberts, 1976; Williams et al., 1985). In fact, the primary amino acid sequence of helix-unwinding protein 1 (UP1) (Williams et al., 1985) isolated from calf thymus or mouse myeloma is nearly identical to the N-terminal fragment of protein A1 and contains N^G,N^G-dimethylarginine (asymmetric) at residue 194. Cobianchi et al. (1988) have overexpressed protein A1 in *Escherichia coli* carrying rat protein A1 cDNA coding sequences, and further purified the recombinant protein A1. They have shown that this recombinant form has no N^G-methylarginine in its primary amino acid sequence but possesses cooperative binding properties to ss nucleic acids.

Specific protein-arginine methylation is catalysed by a group of enzymes called protein methylase I (*S*-adenosyl-L-methionine:protein-L-arginine *N*-methyltransferase, EC 2.1.1.23) with *S*-adenosyl-L-methionine (AdoMet) as the methyl donor (Paik and Kim, 1980; Kim et al., 1990). Specificity of protein methylase I is not only limited to arginine residues of substrate proteins, but to those on the total protein molecule. Thus, myelin basic protein-specific and nuclear protein/histone-specific enzymes have been purified from calf brain, and their differential molecular properties, substrate protein affinities and immunological identities have been characterized (Ghosh et al., 1988). Furthermore, cytochrome *c*-specific protein methylase I has also been identified from *Euglena gracilis* (Farooqui et al., 1985).

Recent studies from this laboratory have shown that the purified nuclear protein/histone protein methylase I can methylate the recombinant protein A1 and the methylation sites for this enzymically [*methyl*-^{3}H]-labelled protein A1 have been identified to be residue-194 arginine of the N-terminal domain in addition to undetermined site(s) at the C-terminal domain (Rajpurohit et al., 1994). Availability of recombinant protein A1 in its unmethylated and methylated forms provided us with an opportunity to study some of their properties and sensitivities toward trypsin and to compare any significant change(s) brought about by the posttranslational arginine methyl-modification on hnRNP protein A1.

MATERIALS AND METHODS

Materials

S-Adenosyl-L-[*methyl*-^{3}H]methionine (specific activity, 78.5 Ci/mmol) was purchased from New England Nuclear, Boston,

Abbreviations used: hnRNP, heterogeneous ribonucleoprotein particle; UP1, helix-unwinding protein 1; AdoMet, *S*-adenosyl-L-methionine: ss, single-stranded; PMSF, phenylmethanesulphonyl fluoride; TPCK, 1-tosylamido-2-phenethyl chloromethyl ketone.

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MA, U.S.A. Carrier ampholytes (pH ranges 7–9 and 8–10) were obtained from Bio-Rad. Phenylmethanesulphonyl fluoride (PMSF), ssDNA-cellulose, AdoMet, pepstatin A and DNA (calf thymus) were all from Sigma Chemical Co. Coliphage MS2-RNA was purchased from Boehringer-Mannheim and 1-tosylamido-2-phenethyl chloromethyl ketone (TPCK)-treated trypsin was from Cooper Biochemicals. All other chemicals were of the reagent grade available from various commercial sources.

Isolation of hnRNP protein A1

Recombinant protein A1, overexpressed in the *E. coli* harbouring the expression vector plasmid pEX11 carrying the protein A1 coding sequence, was grown as described previously (Cobianchi et al., 1988). The protein A1 was then isolated and purified by a DEAE-cellulose and an ssDNA/cellulose column, which were connected in tandem essentially as described (Cobianchi et al., 1988; Rajpurohit et al., 1994).

Purification of protein methylase I

Nuclear protein/histone-protein methylase I was purified from calf brain according to the method described previously (Ghosh et al., 1988). Briefly, the supernatant at 105000 ***g*** was subjected to ammonium sulphate precipitation (40–70 % saturation). The precipitates were then subjected to DE-52 and Sephadex G-200 column chromatographies. The enzymically active fractions were pooled and concentrated by a small DE-52 column (0.5 cm × 1 cm) as described previously (Ghosh et al., 1988; Rajpurohit et al., 1994). The enzyme preparation transferred 90 pmol of methyl-group/min per mg of enzyme protein at 37 °C under the assay condition (Rajpurohit et al., 1994), using protein A1 as the methyl acceptor substrate.

Protein concentration was estimated by Bradford's Coomassie Blue protein reagent (Bradford, 1976).

Preparation of [*methyl*-^{3}H]protein A1

Unmethylated recombinant protein A1 (1.0 mg) was incubated with 120 μM Ado[*methyl*-^{3}H]Met (103 d.p.m./pmol; diluted with unlabelled AdoMet), purified protein methylase I (1.37 mg) and 0.1 M phosphate buffer (pH 7.6) in a total volume of 10 ml for 2 h at 37 °C. After the reaction, the mixture was directly loaded onto an ssDNA cellulose column (1 cm × 5 cm) pre-equilibrated at 4 °C in buffer A [50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 10 mM $Na_2S_2O_5$, 1 mM PMSF, 1 mg/ml pepstatin A] containing 0.3 M NaCl. After extensive washing to remove all unreacted Ado[*methyl*-^{3}H]Met, the column was further washed with five column volumes of buffer A containing 0.4 M NaCl. Subsequently, protein A1 was eluted with buffer A containing 1.0 M NaCl. Peak fractions containing the radio-labelled and -unlabelled protein A1 were pooled and dialysed against buffer A containing 1.0 M NaCl at 4 °C for 3 h to remove any radioactivity carried over non-specifically. The methylated protein A1 thus obtained contained 1.1 mmol–1.9 mol [*methyl*-^{3}H] groups/mol protein A1 (this range of values being obtained in different experiments) and migrated as a single band on SDS/polyacrylamide gel at approximately 34 kDa (cf. Figure 1).

Gradient elution of [*methyl*-^{3}H]protein A1 on ssDNA-cellulose column

Recombinant protein A1 (1.0 mg) was methylated as described above by purified nuclear protein/histone protein methylase I. The incubation mixture was directly loaded onto an ssDNA cellulose column (1 cm × 5 cm) as described above. The column was washed with buffer A containing 0.3 M NaCl and further with two column volumes of buffer A containing 0.5 M NaCl. Then, a gradient consisting of 25 ml of buffer A/0.5 M NaCl in the first chamber and 25 ml of buffer A/0.8 M NaCl in the second was applied. Fractions of 1 ml were collected, A_{280} was measured and an aliquot from each fraction was counted for radioactivity. The fractions containing protein A1 were pooled separately into four different groups as described in the legend of Figure 1 and a 50 μl aliquot of each was subjected to SDS/PAGE.

SDS/PAGE

SDS/PAGE was carried out according to the method of Laemmli (1970) with 15 % acrylamide for the running gel and 3 % for the stacking gel in the presence of 0.1 % SDS. The gel was stained with Coomassie Blue-R250 to visualize protein bands. In order to develop autofluorography, the stained gel was treated with

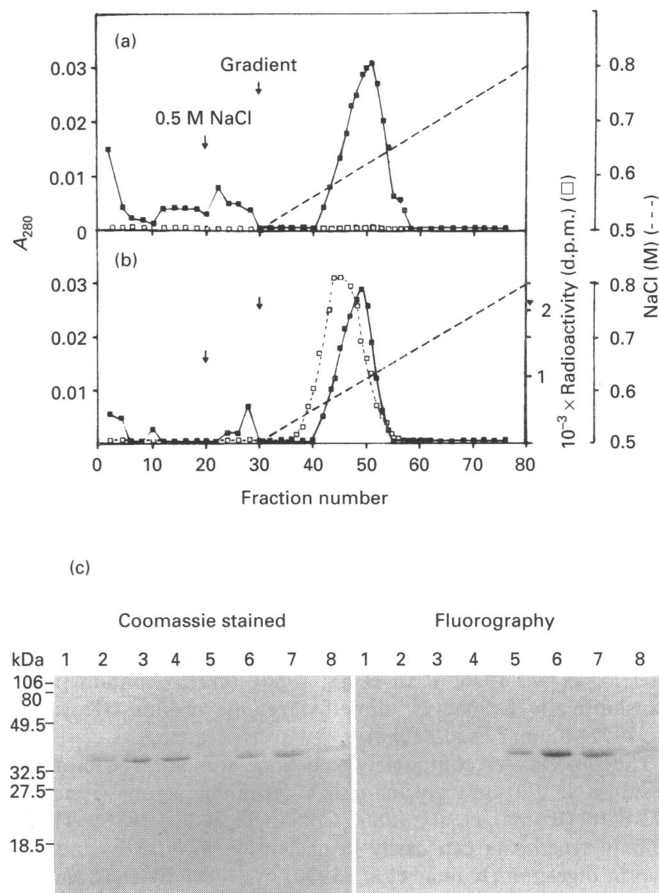

Figure 1 Separation of methylated and unmethylated protein A1 by ssDNA/cellulose chromatography

Protein A1 (1 mg), either unmethylated (**a**) or enzymically [*methyl*-^{3}H]-methylated (**b**) was loaded onto an ssDNA-cellulose column (1 cm × 5 cm) pre-equilibrated in buffer A containing 0.3 M NaCl. The column was first washed with buffer A/0.3 M NaCl and then with buffer A/0.5 M NaCl. Subsequently, a gradient consisting of 25 ml each of buffer A/0.5 M NaCl and buffer A/0.8 M NaCl was applied. Fractions were collected and A_{280} (■) was measured and radioactivity counted (□). Details of the experimental procedures are described in the Materials and methods section. (**c**) Shows SDS/PAGE of ssDNA/cellulose purified protein A1. Lanes 1–4 represent unmethylated protein A1 from (**a**) and lanes 5–8, methylated A1 from (**b**). Each lane represents pooled fractions from the respective chromatography. Samples used for lanes 1 and 5 were the pooled fractions 41–45; lanes 2 and 6, fractions 46–50; lanes 3 and 7, fractions 51–55; and lane 4 and 8, fractions 56–60.

En^{3}Hancer (NEN) for 90 min, washed twice with distilled water for 10 min each and then washed with 5% glycerol for 30 min. The gel was finally dried and exposed to a Kodak X-Omat diagnostic film at −70 °C.

Identification of [*methyl*-^{3}H]amino acid by h.p.l.c.

[*Methyl*-^{3}H]protein A1 was hydrolysed in 6 M HCl at 110 °C for 24 h *in vacuo*. After removal of the acid by extensive washing, amino acids were analysed by h.p.l.c. (Waters Associates) on a strong cation-exchange amino acid analysis column (0.4 cm × 25 cm, Waters Associates) maintained at 59 °C, coupled with the post-column *o*-phthaldialdehyde derivative formation method (Rawal et al., 1992). The elution was effected by isocratic elution with 0.4 M Na-citrate buffer (pH 5.09) at a flow rate of 0.5 ml/min over 160 min. Fractions of 0.5 ml/min were collected and counted for radioactivity in an LKB 1209 RACKBETA liquid scintillation counter.

Isoelectrofocusing of protein A1

Unmethylated protein A1 (2.2 mg) was mixed with [*methyl*-^{3}H]protein A1 (190 μg, 24000 d.p.m.), and electrofocusing was performed according to the method of Vesterberg (1971) on a linear gradient of sorbitol (Park et al., 1989). Electrofocusing was carried out at 2–4 °C at 800 V for 22 h. Fractions of 1.2 ml were collected and pH and A_{280} as well as radioactivity were determined for each fraction.

Fluorescence studies

Fluorescence and quenching measurements were carried out on a Perkin-Elmer LS-5 fluorescence spectrophotometer with an excitation wavelength of 285 nm with a slit-width of 3 nm and emission at 310 nm with a slit-width of 5 nm. The emission maxima was observed at 310 nm. Binding of MS2-RNA to protein A1 was carried out at a protein concentration of 170 nM in 10 mM Tris/HCl (pH 7.5), 1 mM EDTA and increasing concentrations of MS2-RNA (based on the number of nucleotide bases) in a total volume of 2 ml. The non-cooperative binding association constants were calculated from double reciprocal plots as described (Spicer et al., 1979). The concentration of free MS2-RNA was determined by subtracting the concentration of bound protein from the initial MS2-RNA concentration. The concentration of bound protein was determined from the ratio of the fluorescence change with respect to the MS2-RNA concentration to the maximum fluorescence change ($\Delta F_{\mathrm{initial}}/\Delta F_{\mathrm{maximum}}$ × initial protein concentration). Double reciprocal plots were constructed in which $1/\Delta F_{\infty}$ was plotted versus $1/[\text{MS2-RNA}]_{\mathrm{free}}$ with the intercept y giving the maximum fluorescence change and the slope $1/K\Delta F_{\infty}$ allowing the measurement of the association constant (K_a).

Controlled digestion of protein A1 with trypsin

Methylated or unmethylated protein A1 was incubated with trypsin under controlled conditions in the presence or absence of MS2-RNA or ssDNA. Protein A1 (12.4 μg) was digested with TPCK-treated trypsin at a ratio of 1:2500 (trypsin:protein) in 10 mM Tris/HCl (pH 8.0)/1 mM EDTA/0.08 M NaCl in a total volume of 100 μl at 22 °C for 0, 2.5, 5 and 10 min. The ratio of protein:nucleic acid (both MS2-RNA or ssDNA) was 1:12. The reaction was stopped by the addition of 50 μl of 3× Laemmli's buffer and the mixture was subjected to SDS/PAGE. Protein bands visualized by Coomassie Blue staining were then quantitatively analysed by tracing the bands with an LKB Ultroscan × laser densitometer.

RESULTS

Methylated and unmethylated protein A1 on ssDNA–cellulose chromatography

Enzymically methylated recombinant hnRNP protein A1 can be easily prepared on an ssDNA–cellulose column by first removing unreacted Ado[*methyl*-^{3}H]Met which does not adsorb on the column and by subsequently eluting protein A1 with 1.0 M NaCl buffer. It was observed during purification, however, that the [*methyl*-^{3}H]protein A1 had a tendency to elute before the A_{280} peak from the ssDNA-cellulose column. Thus, by applying an NaCl gradient (0.5 M–0.8 M), it was possible to separate the major radioactive peak from the A_{280} peak with a difference of 4–5 tubes (Figures 1a and 1b); the NaCl concentration required to release the bound [*methyl*-^{3}H]protein A1 was shown to be 0.59 M whereas that for the A_{280} peak (mixture of methylated and unmethylated) was 0.63 M. In order to examine any molecular alteration, such as proteolysis occurring during the methylation reaction, the protein peaks in different fractions were

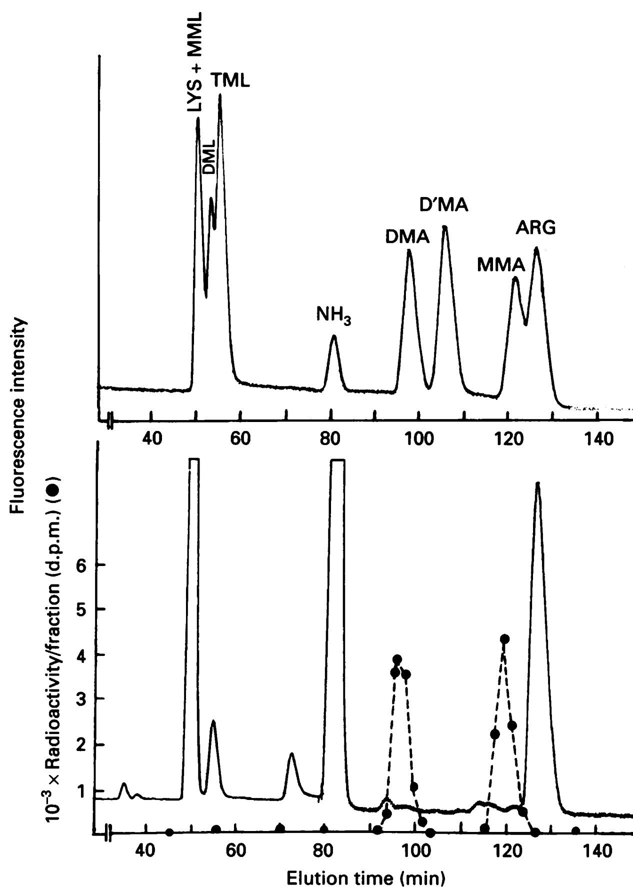

Figure 2 H.p.l.c. analysis of [methyl-^{3}H]amino acid

Enzymically methylated [*methyl*-^{3}H] protein A1 was hydrolysed in 6 M HCl. The hydrolysate was analysed by h.p.l.c. using an amino acid analysis column (Waters Associates) as described in the Materials and methods section. Fractions (0.5 ml/min) were collected and counted for radioactivity (●). Abbreviations used are ARG, arginine; MMA, N^G-monomethylarginine; D′MA, N^G,N^{G}-dimethylarginine (symmetric); DMA, N^G-dimethylarginine (asymmetric); TML, ε-*N*-trimethyllysine; DML, ε-*N*-dimethyllysine; MML, ε-*N*-monomethyllysine; and LYS, lysine.

analysed by SDS/PAGE. As shown in Figure 1(c), proteins from all fractions showed identical mobility at approximately 34 kDa, evidenced by both Coomassie-staining and fluorography. This confirmed that no molecular size alteration occurred during the reaction and that the differential elution profile is not due to a possible degradation of the protein A1, but rather to the side-chain arginine-methylation. Furthermore, u.v. absorption maxima of methylated and unmethylated protein A1 were both at 278.5 nm, determined using a Beckman DU-7 spectrophotometer, suggesting that no significant conformational alteration occurred near tyrosine residues (results not shown).

A closer look at the Coomassie-staining bands (Figure 1c) of the unmethylated (lanes 1 and 2; obtained in Figure 1a) and the methylated fractions (lanes 5 and 6; obtained in Figure 1b) showed that while the lane 5 (pooled fractions 41–45) showed a distinct protein band, the comparable fraction of the unmethylated protein A1 in lane 1 did not show any band, indicating a lag in the elution of unmethylated protein A1. Furthermore, in comparing the intensities of the Coomassie-staining and fluorography of the methylated fractions (lanes 6 and 7), it is quite obvious that the radiomethyl-incorporation was much stronger in lane 6 than in lane 7 whereas the Coomassie-staining of lane 7 was darker than that of the lane 6. This observation again confirms the above contention that the [*methyl*-^{3}H]protein A1 elutes from the ssDNA-cellulose column before the unmethylated protein. An attempt has been made to separate completely the methylated A1 from the unmethylated by applying a shallower salt gradient during ssDNA–cellulose chromatography, however without success; neither has it been possible to separate protein A1 containing only monomethyl- or dimethylarginine.

Based on the extent of [*methyl*-^{3}H]group incorporation and the amounts of N^{G}-methylarginine derivatives formed (Figure 2), it was calculated to contain 1.45 mol methylarginine per mol of protein A1 (see below). It is quite probable that the [*methyl*-^{3}H]protein A1 preparation herein is heterogeneous in respect to the kind of N^{G}-methylarginine derivatives and the site(s) of methylation. Notwithstanding, since this methylated recombinant protein A1 preparation, *in vitro*, contained 1.45 mol methylarginine derivatives in contrast to 3.1 mol of dimethylarginine per mol of protein A1 isolated from HeLa cells (Kumar et al., 1986), it was felt worthwhile to compare some of the physicochemical properties of this *in vitro* methylated protein A1 with the totally unmethylated recombinant protein A1.

Identification of methylated amino acid

The methylated amino acids in [*methyl*-^{3}H]protein A1 have been examined by h.p.l.c. analysis (Figure 2). The radioactivity is exclusively associated with N^{G}-monomethylarginine (52%) and N^{G},N^{G}-dimethylarginine (asymmetric) (48%). Neither the formation of N^{G},N'^{G}-dimethylarginine (symmetric) nor any of the methylated lysine derivatives is seen, demonstrating the high degree of enzyme specificity. Based on the specific radioactivity of the [*methyl*-^{3}H]group, 1.9 mol of methyl-groups were found to be taken up by one mol of protein A1. Since the [*methyl*-^{3}H]protein A1 contained both N^{G}-monomethylarginine (52%) and N^{G},N^{G}-dimethylarginine (48%) (Figure 2), and the latter isomer incorporated two mol of methyl groups, the extent of methylation can be calculated as 1.45 mol per mol of [*methyl*-^{3}H]protein A1. This level of methylation amounts to approximately 47% of the level *in vivo* where 3.1 mol of N^{G}-dimethylarginines were present in HeLa cell protein A1 (Kumar *et al.*, 1986). The precise location of all three methylarginines in the protein A1 have not yet been clarified, except residue-194

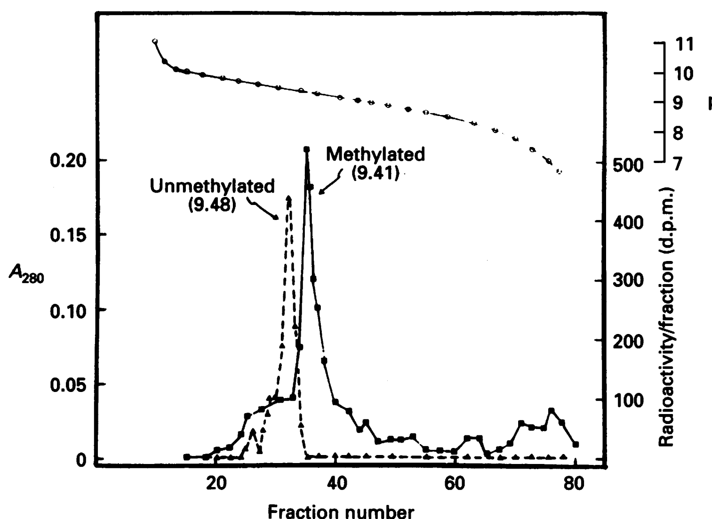

Figure 3 Isoelectrofocusing chromatography of methylated and unmethylated protein A1

A mixture of [*methyl*-^{3}H]protein A1 (190 μg, 24000 d.p.m.) and unmethylated protein A1 (2.2 mg) was applied on an isoelectrofocusing column as described in the text: (▲) unmethylated and (■) methylated protein A1.

(Williams et al., 1985; Rajpurohit et al., 1994), and in the C-terminal domain (Rajpurohit et al., 1994).

Change of pI value of protein A1 by methylation

In order to discern possible charge alteration on protein A1 structure resulting from the methylation, the pI values of unmethylated and enzymical methylated protein A1 were determined by isoelectrofocusing. Figure 3 indicates that the pI of the former was 9.48 and that of the latter 9.41. Although the difference in the pI values (0.07 unit) appears not to be drastic, this difference is extremely significant, since both [*methyl*-^{3}H]-labelled and unmethylated protein A1 were run simultaneously. The lowering of the pI of protein A1 by methylation (less basic) may have contributed to the lower binding of the protein to the highly anionic ssDNA cellulose column (Figure 1).

Effect of methylation on the interaction of protein A1 with nucleic acids

To compare binding-site size of coliphage ssMS2-RNA with methylated and unmethylated protein A1, the decrease in the protein fluorescence was measured in the presence of increasing concentrations of MS2-RNA at a fixed amount of proteins (Figure 4). The maximum quenching observed with unmethylated protein A1 was 45% whereas that with the methylated protein was 40.3%. This indicates a lower binding with the methylated protein A1. However, the binding-site size, calculated based on the ratio of protein to nucleotide at the point where the initial slope of the fluorescence quenching intersects the fully quenched fluorescence, was not significantly different; 54 nucleotide residues with the unmethylated and 58 residues with methylated protein A1, respectively. These values are more than four times larger than the binding-site size of the synthetic polynucleotides, poly(ethanoadenylate), with unmethylated protein A1 (12

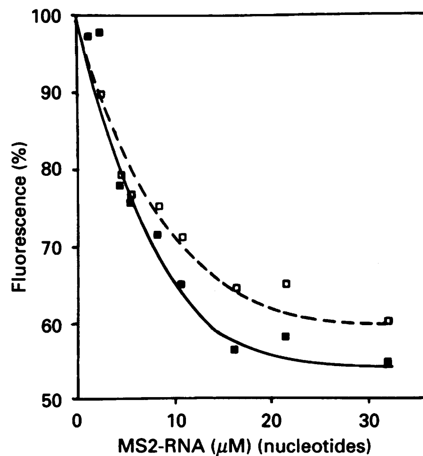

Figure 4 Stoichiometric titration of methylated and unmethylated protein A1 with coliphage MS2-RNA

Protein A1 (170 nM), either methylated (□) or unmethylated (■), was titrated with increasing concentrations of MS2-RNA in 10 mM Tris/HCl (pH 7.5) and 10 mM NaCl. Values for fluorescence are expressed as a percentage of initial fluorescence.

nucleotides) (Cobianchi et al., 1988). This difference could be attributed to the preference of protein A1 for the natural polynucleotides such as coliphage MS2-RNA which is known to have a greater affinity toward protein A1 (Kumar et al., 1986). Although the methylated protein A1 tends to exhibit a decreased binding-capacity toward MS2-RNA than the unmethylated, the non-cooperative association constants between the unmethylated (5.75×10^6 M^{-1}) and methylated protein A1 (4.63×10^6 M^{-1}) were not significantly different. The concentration of NaCl required to reverse 50% of the binding of MS2-RNA to protein A1 was examined and found to be 235 mM for the unmethylated and 200 mM for the methylated protein A1, respectively.

Change in trypsin sensitivity of protein A1 by methylation and its effect in the presence of nucleic acids

Protein A1 is a two-domain molecule which can be easily cleaved by limited tryptic digestion, resulting in the generation of the 24 kDa N-terminal and the 10 kDa C-terminal fragments (Kumar et al., 1990). While the 24 kDa fragment is relatively stable, the 10 kDa fragment is extremely sensitive to trypsin and degrades further into smaller peptides. We have examined the relative rate of trypsin digestibility of methylated and unmethylated A1. As shown in Figure 5, the methylated A1 was completely digested in 5 min under the controlled conditions (Figure 5a and b, both 5 min), while a small peak of undigested protein, 34 kDa, still remained after 10 min in the case of the unmethylated protein (Figure 5a and b, both 10 min), suggesting that the unmethylated A1 is relatively resistant toward trypsin. It has been reported earlier (Kumar et al., 1990; Rajpurohit et al., 1994) that trypsin cleaves Arg-196 of protein A1 which is located two residues upstream of the methylated Arg-194 residue. Obviously, the trypsin cleavage site in both methylated and unmethylated protein A1 is expected to be the same, residue 196, and is different from the methylation site at Arg-194 which is known to resist trypsin digestion (Brostoff and Eylar, 1971; Lischwe et al., 1985a; Kumar et al., 1990).

The differential trypsin sensitivity between the methylated and unmethylated protein A1 was further studied in the presence of ssRNA or ssDNA. The protein to nucleic acid ratio (1:12) was

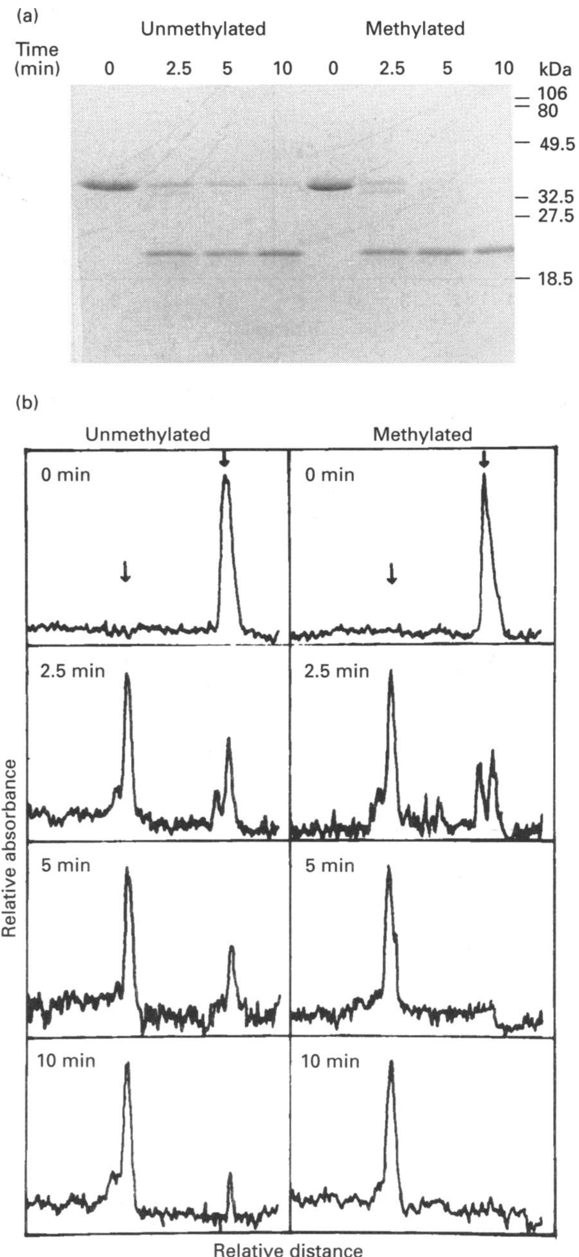

Figure 5 Controlled trypsin digestion of methylated and unmethylated protein A1

Either methylated or unmethylated protein A1 (each 12.4 μg) was digested with trypsin (trypsin:protein ratio, 1:2500) for 0 min, 2.5 min and 10 min at 22 °C as described in the Materials and methods section. The digestion mixtures were then subjected to PAGE (**a**) and the Coomassie Blue stained gel was quantified by densitometric tracing (**b**). The methylated protein A1 contained 1.9 mol [*methyl*-^{3}H]group per mol protein. The arrows indicate approximate molecular mass: 24 kDa on the left and 34 kDa on the right.

adopted from the work of Cobianchi et al. (1988) and Kumar et al. (1986) in which the maximum binding of protein A1 to polynucleotides or ssDNA was achieved at that ratio. The results have been expressed as percent peak area of the undigested protein as a function of digestion period, determined by densitometric tracing of SDS/polyacrylamide gels (Figure 6). Based on a semi-log plot using the values from the Figure, the time required to digest 50% of protein A1 under the experimental

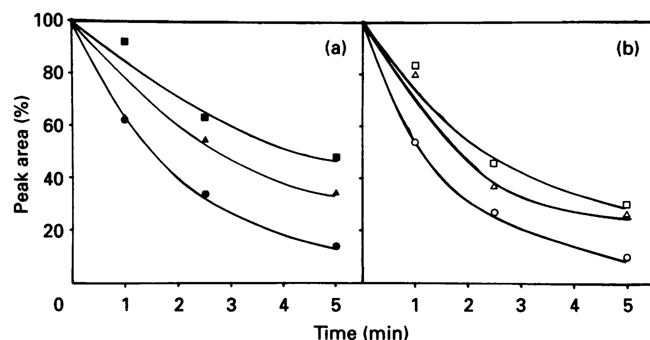

Figure 6 Trypsin digestion of methylated and unmethylated protein A1 in the presence of MS2-RNA and ssDNA

The experimental conditions are the same as in Figure 5 except that the trypsin digestion was carried out in the presence of MS2-RNA (■,□), ssDNA (▲,△) or no nucleic acid (●,○). The ratio of protein:nucleic acid was 1:12 (based on the number of nucleotides). Values are the average of two independent determinations. Solid symbols represent unmethylated (**a**) and open symbols, methylated (**b**) protein A1.

Table 1 Differences in physicochemical properties between recombinant unmethylated and arginine-methylated hnRNP Protein A1

Values are averages of two independent experiments.

Property	Unmethylated	Methylated
pI	9.48	9.41
[NaCl] to release from ssDNA–cellulose	0.63 M	0.59 M
ssMS2-RNA binding:		
K_a	5.75×10^6 M^{-1}	4.63×10^6 M^{-1}
Binding size	54 nucleotides	58 nucleotides
[NaCl] to reverse 50% binding	235 mM	200 mM
Trypsin sensitivity [50% digestion (min)]		
No addition	1.63	1.31
+ssMS2-RNA	4.30	2.41
+ssDNA	2.74	2.00

conditions used was calculated and is summarized in Table 1. In the absence of nucleic acid, the $t_{\frac{1}{2}}$ was 1.63 min for the unmethylated protein A1 (Figure 6a), whereas it was 1.31 min for the methylated (Figure 6b). In the presence of ssMS2-RNA, however, the $t_{\frac{1}{2}}$ difference was much more prominent; $t_{\frac{1}{2}}$ was 4.3 min for the unmethylated and 2.41 min for the methylated protein. We have also studied MS2RNA at a ratio of 1:55 and found very similar results to those obtained at a ratio of 1:12. It should be mentioned that the protein A1 is known to bind MS2RNA with much greater affinity compared with all other polynucleotides such as DNA and poly(ethanoadenylate) (Kumar et al., 1986). The effect of ssDNA was not as great as in the case of ssRNA (2.74 min versus 2.0 min for the unmethylated versus methylated). The above results thus indicate that protein A1 upon arginine-methylation becomes more susceptible to trypsin and that the sensitivity is further enhanced by the presence of ssRNA, which complexes with the methylated protein A1 less tightly than with the unmethylated.

DISCUSSION

Among many proteins known to contain N^G-methylarginine residues (Reporter and Corbin, 1970; Brostoff and Eylar, 1971; Karn et al., 1977; Lischwe et al., 1985a,b; Wang et al., 1982), protein A1 is one of the most highly methylated proteins *in vivo*, containing 3.1 mol of dimethylarginine per mol of the protein (Kumar et al., 1986), which makes it an ideal model molecule to study the structure–function relationship of this posttranslational arginine side-chain modification reaction. Maximally *in vitro*, we were able to methylate the recombinant unmethylated protein A1 by the purified nuclear protein/histone protein methylase I up to 1.45 mol methyl/mol protein. Further attempts to increase the methylation *in vitro* to the level *in vivo* were unsuccessful. Using this preparation, which contained about 47% of the level of methylation *in vivo* [3.1 mol N^G,N^G-dimethylarginine (asymmetric)/mol of protein A1 (Kumar et al., 1986)], the effect of enzymic methylation on several characteristics of the protein were investigated. As summarized in Table 1, all the parameters examined, such as the change in pI value, the binding properties of the ssDNA/cellulose column, the fluorescence quenching in the presence of ssRNA, and the trypsin sensitivity in the presence of MS2-RNA, demonstrated that the methylated protein A1 exhibited lower binding-capacity toward ss nucleic acids than the unmethylated. In other words, the enzymic arginine-methylation rendered the protein A1 less basic and/or more hydrophobic than the unmethylated, thus making the N^G-methylarginine-containing protein A1 interact less tightly with nucleic acids than the unmethylated species. Related to this is a recent study by Connell et al. (1993) on the isolation of three RNA binding motifs. These motifs which had been bound to an L-arginine affinity column were eluted with arginine, but much less efficiently when N^G-methylarginine was used. This finding is consistent with our present observation that the N^G-methylarginine-containing protein A1 interacted with ssRNA less tightly than with the unmethylated A1.

The pI value of protein A1 was shown to be lowered by 0.07 pH unit following its arginine-methylation (9.48 for unmethylated versus 9.41 for methylated; Figure 3). This decrease of pI by enzymic arginine-methylation appears to be quite contradictory to the reported values for free N^G-methylated arginines [10.77 for dimethylarginine (asymmetric); 10.54 for methylarginine; and 10.02 for arginine (Paik et al., 1983)]. This seemingly contradictory observation was also found in the cases of other posttranslationally methylated proteins. For example, the enzymic trimethylation of lysine residues of cytochrome *c* decreased its pI from 10.03 to 9.49 and that of calmodulin from 4.04 to 3.97 (Park et al., 1988, 1989). These unanticipated changes in pI values suggest that, in addition to a local effect, some overall conformational rearrangement of the protein structure occurred as a consequence of the side-chain modification (Paik and Kim, 1992).

One of the most notable findings in this paper is that the methylated protein A1 was much more sensitive to mild trypsin digestion than the unmethylated (Table 1 and Figure 6); the $t_{\frac{1}{2}}$ values for unmethylated and methylated protein A1 in the presence of MS2-RNA were 4.30 min and 2.41 min, respectively. This reaction cleaves protein A1 at residue 196 to generate UP1 polypeptide (corresponding to the N-terminal domain of A1); the analogous reaction is known to occur in nuclei by a trypsin-like protease (Pandolfo et al., 1985). The UP1 isolated from the eukaryotic cells were shown to contain N^G-methylarginine at residue 194 (Williams et al., 1985), and the same arginine residue was also methylated when the recombinant unmethylated protein A1 was methylated *in vitro* by protein methylase I (Rajpurohit et al., 1994). Surprisingly, however, when the unmethylated N-terminal fragment (residues 1–196) was first prepared *in vitro* by trypsin and then subjected to enzymic methylation, the fragment was no longer a methyl-acceptor for protein methylase I

(R. Rajpurohit, W. K. Paik and S. Kim, unpublished work). In view of these observations, it is tempting to speculate that the enzymic methylation of Arg-194, which is located two residues downstream from the trypsin cleavage site, may serve as a modification signal to enhance the formation of UP1 *in vivo*. It should be mentioned that UP1 and its analogous proteins are widely distributed among eukaryotic cells (Herrick et al., 1976; Williams et al., 1985; Riva et al., 1986; Kumar et al., 1990) and UP1 is a very effective activator for α DNA polymerase; while protein A1 stimulated only 1.5-fold, UP1 enhanced the polymerase activity 5.0–6.2-fold (Riva et al., 1986). At this juncture, Riva et al. (1986) have suggested a possible alternative biological role of UP1 in DNA synthesis other than its RNA binding property, and have pointed out that UP1 may not be a mere degradation product of hnRNP protein A1.

In addition to the above biological function, the enzymic arginine-methylation of protein A1 might facilitate the importation of hnRNP protein into the nucleus. This is based on the following two considerations. First, over 90% of nuclear protein/histone protein methylase I was found to be present in the cytosol of rat liver (Rajpurohit et al., 1994) where the nascent protein A1 polypeptide is synthesized, and, secondly, it has been demonstrated that the enzymic lysine-methylation of yeast cytochrome *c* enhances its importation into the mitochondria (Park et al., 1987).

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