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Immunoglobulin Can Be Functionally Regulated by Protein Carboxylmethylation in Fc Region

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Protein carboxylmethylation methylates the free carboxyl groups in various substrate proteins by protein carboxyl *O*-methyltransferase (PCMT) and is one of the post-translational modifications. There have been many studies on protein carboxylmethylation. However, the precise functional role in mammalian systems is unclear. In this study, immunoglobulin, a specific form of γ-globulin, which is a well-known substrate for PCMT, was chosen to investigate the regulatory roles of protein carboxylmethylation in the immune system. It was found that the anti-BSA antibody could be carboxylmethylated *via* spleen PCMT to a level similar to γ-globulin. This carboxylmethylation increased the hydrophobicity of the anti-BSA antibody up to 11.4%, and enhanced the antigen-binding activity of this antibody up to 24.6%. In particular, the Fc region showed a higher methyl accepting capacity with 80% of the whole structure level. According to the amino acid sequence alignment, indeed, 7 aspartic acids and 5 glutamic acids, as potential carboxylmethylation sites, were found to be conserved in the Fc portion in the human, mouse and rabbit. The carboxylmethylation of the anti-BSA antibody was reversibly demethylated under a higher pH and long incubation time. Therefore, these results suggest that protein carboxylmethylation may reversibly regulate the antibody-mediated immunological events via the Fc region.

Key words: Protein carboxylmethylation, Immunoglobulin, Structure and function, Protein carboxyl-*O*-methyltransferase

INTRODUCTION

Protein carboxylmethylation (PCM) is a biochemically inefficient reaction compared with protein phosphorylation. Protein phosphorylation requires 1 ATP molecule per reaction (Krebs and Beavo, 1979), whereas PCM requires 12 ATP molecules (Hrycyna and Clarke, 1993). Nevertheless, PCM is still highly conserved in biological systems, suggesting that it plays an important role in some special functions or functions. This modification is catalyzed by protein carboxyl *O*-methyltransferase [EC 2.1.1.24] [PCMT, also known as protein methylase II], which transfers a methyl group from *S*-adenosyl-L-methionine (AdoMet) to a free carboxyl group in a methyl accepting polypeptide (Paik and Kim, 1990). Consequently, a change in polarity by carboxylmethylation can cause a conformational change that regulates important molecular functions of the substrate proteins (Paik and Kim, 1992), as in other post-translational modification reactions. Furthermore, negative physiological regulations by a change in pH, activation of methylesterase (demethylating enzyme) (Veeraragavan and Gagnon, 1989), and the existence of inhibitory molecules such as natural proteinacious inhibitors (Hong *et al.*, 1986; Park *et al.*, 1993; Kwon *et al.*, 1994; Seo *et al.*, 2002) and *S*-adenosyl-L-homocysteine (AdoHcy) (Paik and Kim, 1990) appear to support the importance of this modification.

To date, PCMT is classified into five different classes (Aswad, 1989; Hrycyna and Clarke, 1993), according to

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the target amino acids to be carboxylmethylated. Each class of PCMT plays a unique role in the post-translational modifications. Therefore, it is important to identify the carboxylmethyl esters in order primarily to understand the role of PCM in the target cells or tissues. The first class (E.C. number 2.1.1.80) has been identified only in chemotatic bacteria. This enzyme catalyzes the formation of relatively stable L-glutamic acid γ-methylesters on discrete chemoreceptor proteins (Barten and O'Dea, 1990; Koshland, 1988; Clarke, 1985). The second class (E.C. number 2.1.1.77) is believed to participate in repairing aged proteins *via* the selective carboxylmethylation of atypical L-isoaspartyl or D-aspartyl residues (Ingrosso *et al.*, 2000; Farrar and Clarke, 2002; Clarke, 2003). The third class (EC 2.1.1.24) is a widely distributed enzyme, which methylates aspartyl and glutamyl residues in proteins (Aswad, 1989), demonstrates a low degree of substrate specificity, and catalyzes reactions that are markedly sub-stoichiometric (Kloog *et al.*, 1982). The physiological significance of these latter reactions is uncertain. The fourth class has been described in eukaryotes (Clarke *et al.*, 1988), and methyl-esterifies the α-carboxyl groups of the C-terminal cysteine residues in some signaling proteins including the ras oncogene protein and several related guanine nucleotide-binding proteins (Hrycyna and Clarke, 1993; Yamane *et al.*, 1991). With prenylation or farnesylation, class IV PCMT-mediated modification has been reported to critically regulate the membrane targeting of these proteins (Hrycyna and Clarke, 1993). The fifth class (class V) of PCMT carries out novel modification reactions in which the a-carboxyl group of the C-terminal leucine in cytosolic proteins, such as in protein phosphatase 2A, is methylated (Xie and Clarke, 1994; Lee and Stock, 1993; Vafai and Stock, 2002). These two classes of PCMTs catalyze the generation of basestable CMEs. Currently, class IV- or class V-mediated carboxymethylation reactions appear to be a new therapeutic intervention for treating cancer, pancreatic β-cell impairment, and Alzheimer's disease (Hrycyna and Clarke, 1993; Zolnierowicz, 2000; Kowluru and Amin, 2002; Vafai and Stock, 2002; Winter-Vann *et al.*, 2003).

Thus far, the functional roles of protein carboxylmethylation on the secretion-excitation processes in the mammalian pancreas, neurons, and brain (Diliberto *et al.*, 1976; Diliberto and Axelrod, 1976; Kowluru *et al.*, 1996; Kowluru and Amin, 2002) have been only been suggested. However, there is little evidence that can explain the function of PCMT in the immune system or immunological components such as immunoglobulin and complement. Based on previous results showing that 1) γ-globulin is a well-known substrate for PCMT (Kim *et al.*, 1994), 2) this enzyme has been found to be highly active in the spleen (Cho *et al.*, 2004), 3) the chemotactic motility of immune cells is regulated by protein carboxylmethylation (Leonard *et al.*, 1978; Pike *et al.*, 1978; Rodriguez *et al.*, 1991), 4) there is clear involvement of protein carboxylmethylation in lipopolysaccharide-treated B cells (Law *et al.*, 1992), and 5) the failure of normal PCMT function leads to T cell hyperproliferation and the production of autoantibodies (Doyle *et al.*, 2003). Hence, protein carboxylmethylation may be one of many important cellular phenomena relevant to various immune responses. Therefore, this study examined the regulatory role of protein carboxylmethylation on the immunological component by examining the biochemical and immunological features of carboxylmethylated immunoglobulin.

MATERIALS AND METHODS

Materials

Bovine γ-globulin, cytochrome C, gelatin, albumin and papain were purchased from Sigma Chemical Co. (St. Louis, MO). Radiolabeled *S*-adenosyl-L-[methyl-^{14}C]- methionine ([methyl-^{14}C] AdoMet, specific activity : 63 mCi/mmol) was from Amersharm (Arlington Heights, IL). 4,4'-bis-1-phenylamino-8-naphthalene sulfonate (Bis-ANS) was a product of Molecular Probes (Eugene, OR). *O*-Phenylenediamine was obtained from Pierce (Rockford, IL). Other chemicals not mentioned specifically were of the highest grade commercially available.

Purification of Protein carboxylmethyltransferase (PM II)

Fresh porcine spleen was purchased from a local slaughterhouse. The spleen PCMT (psPCMT) was purified using the procedure established in our laboratory (Kim *et al.*, 1994; Cho *et al.*, 2004).

Enzyme activity and methyl accepting capacity (MAC)

The enzymatic activity of the psPCMT and its methyl accepting capacity were determined using the methanol extraction method described in the literature (Kim and Paik, 1970). Briefly, fifty microliters of a citrate-phosphate buffer (pH 6.0), 20 μL of γ-globulin or anti-BSA antibody fractions [whole body, and Fab and Fc fractions (final 125 μM)] and 20 μL of the enzyme fraction (final 2.25 μg) were added in a final 100 μL. The reaction was begun by adding 10 μL of [methyl-^{14}C] AdoMet (63 mCi/mmol in sulfuric acid), and allowed to proceed for 15 min. the reaction was quenched by adding 100 μL of a borate buffer, pH 11.0. The mixture was incubated again at 37°C for 5 min and then cooled in an ice bucket. 1 mL of isoamyl alcohol was added and the mixture was stirred vigorously for 15 seconds on a vortex mixer. The isoamyl alcohol layer was separated by centrifugation in a tabletop

centrifuge (Hanil, Seoul, Korea) for 2 min. After centrifugation, a 700 μL portion of the isoamyl alcohol extract was transferred into a 20 mL glass liquid scintillation vial containing 5 mL of a scintillation cocktail and the radioactivity was counted using a liquid scintillation counter (Pharmacia, Sollentuna, Sweden). The protein concentration of the purified enzyme was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976).

Preparation of anti-BSA antibody

To investigate the role of carboxylmethylation in modulating the functional roles of immunoglobulin, polyclonal Anti-BSA antisera were prepared from rabbits, which had been immunized with BSA. The Anti-BSA antibody fraction was purified by salt precipitation and Protein A-affinity chromatography.

Partial digestion of rabbit anti-BSA antibody with papain

The Fab fraction was obtained by proteolysis of the whole immunoglobulins by papain (Portei, 1959). The papain and antibody was mixed together at a ratio of 1 : 50 by weight. The anti-BSA antibodies (14 mg) and papain (0.28 mg) were dissolved in 5.5 mL of 10 mM phosphate-buffered saline (PBS, pH 8.0), containing 20 mM EDTA and 20 mM cysteine. The solution was incubated at 37°C for 1 h. After incubation, the reaction was quenched by dialysis. The degree of digestion was monitored by SDS-PAGE, and various fractions were separated on a BSA-Sepharose column. The unbound fractions (Fc fractions) were pooled and concentrated by ultrafiltration. The eluates of the adsorbed protein (Fab fragment) were collected.

Enzyme linked immunosorbent assay (ELISA)

The reactivity of immunoglobulin was measured by an ELISA assay using the secondary (peroxidase-labeled goat anti-rabbit IgG) antibody (Ausubel *et al.*, 1996). For pre-coating, BSA was diluted in PBS (pH 7.4) to a concentration of 1 mg/mL. Unless otherwise stated, all the reagents were diluted in PBS.

Sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-polyacrylamide electrophoresis was performed using the method reported by Laemmli with 12% acrylamide for running gel and 5% for the stacking gel at a constant current of 40 mA for 1 h at room temperature (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue for 2 h, and then destained by soaking in a mixture containing 30% methanol and 10% acetic acid.

Fluorescence spectrophotometry

Unless indicated otherwise, all determinations and experiments were performed using 3 mL of 20 mM sodium phosphate buffer at pH 8.0. The fluorescence measurements were made using a fluorescence spectrophotometer (Hitachi F-4010, Tokyo, Japan) operated in scan mode, at a scan speed 240 nm/min with 0.5 seconds for the response using a 5 nm band-pass for excitation (wavelength : 390 nm), and 10 nm for emission (wavelength : 500 nm),. Unless stated otherwise, all emission scans were observed with excitation at 395 nm. The fluorescence intensity of Bis-ANS bound to the anti-BSA antibody was monitored by adding increasing amounts of Bis-ANS (0.1-100 μM) to a cuvette (1×1 cm) containing a fixed concentration of anti-BSA antibodies (5 μM). The observed fluorescence was corrected for the dilution factors.

RESULTS

Preparation of anti-BSA antibody

The antibodies newly synthesized *in vivo* were freshly prepared by obtaining the antiserum from BSA-immunized rabbits and purifying the anti-BSA antibody using several purification processes [ammonium precipitation and protein A affinity column chromatography (Fig. 1A)] using conventional purification methods. The purity of the purified anti-BSA antibody was confirmed by SDS-PAGE under reducing or non-reducing conditions. Fig. 1B shows that the final fraction after affinity chromatography had a single band at 150 kDa (lane 3) under non-reducing conditions, while two bands (53 and 23 kDa) were observed under reducing conditions (lane 4). The antigen-binding activity of the final fraction was also drastically increased. Therefore, the purified anti-BSA fraction had a 30-times higher binding activity than the antiserum level (Fig. 1C).

Anti-BSA antibody can be carboxylmethylated by spleen PCMT

In order to investigate the protein carboxylmethylation of the anti-BSA antibody, the methyl accepting capacity of the purified anti-BSA antibody was examined using a methanol forming method (Kim and Paik, 1970). γ-globulin (11.9 unit/mg), a well-known substrate protein for PCMT, and anti-BSA antibody (11.8 unit/mg) showed a similar methyl accepting capacity (Table I). In contrast, the carboxylmethylation capacity of these proteins was higher than that of another proteins (pepsin, cytochrome C and gelatin). This suggests that the anti-BSA antibody is a good substrate protein for PCMT. Therefore, this anti-BSA antibody solution was used in further evaluations.

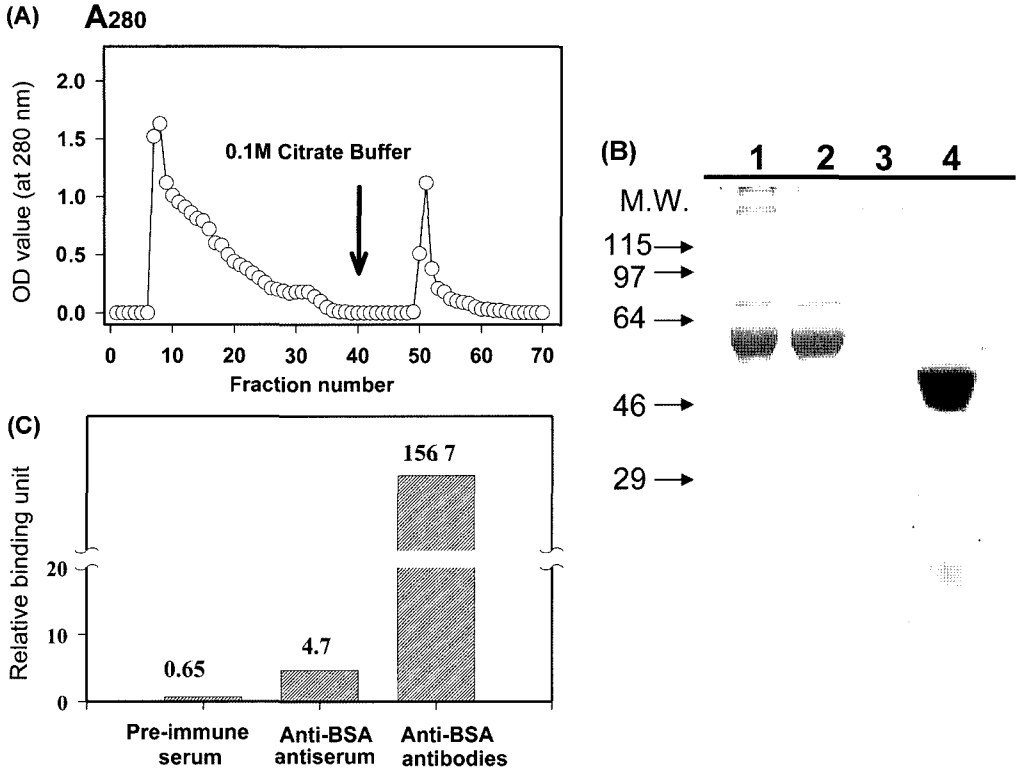

Fig. 1. Preparation of the anti-BSA antibody from anti-serum. (A) Chromatographic patterns of dialyzed suspensions from rabbits on a Protein A-affinity column. The dialyzed suspensions were applied to a Protein A-affinity column (0.5×12 cm) and chromatographed with a 20 mM phosphate buffer, pH 7.3. Elution of the adsorbed protein was carried out with a 100 mM citrate buffer, pH 2.8. (B) SDS-PAGE of the purified anti-BSA antibodies. SDS-PAGE was carried out in 12% acrylamide gels, as described in the materials and method section. The molecular mass standards (β-galactosidase, 115000; Phosphorylase B, 97400; Bovine albumin, 64000; Egg albumin, 46000; carbonic anhydrase, 29000) were used. Lane 1: anti-BSA antisera; Lane 2: unbinding fraction; Lane 3: binding fraction which is thought to be anti-BSA antibodies; and Lane 4: reducing condition. (C) ELISA of purified anti-BSA antibodies. ELISA was carried out as reported in the method section. Anti-sera (1 : 1000) and purified antibody (5 mM) were used. The absorbance was measured at 490 nm of the wavelength.

Table I. Methyl accepting capacity of anti-BSA antibodies

Substrate (125 μM)	Methyl accepting capacity (unit[1]/mg)
anti-BSA antibody	11.8
γ-globulin	11.9
pepsin	3.1
cytochrome C	6.8
gelatin	7.2

[1]Methyl accepting capacity was measured using the methanol extraction method. One unit of the MAC is defined as the amount of substrate that catalyzed the transfer of one pmole of methyl-^{14}C groups incorporated in the substrate proteins per minute.

Hydrophobicity change of carboxylmethylated anti-BSA antibody

The carboxylmethylation of the anti-BSA antibody was confirmed by measuring the change in hydrophobicity using Bis-ANS. As shown in Fig. 2, the carboxylmethylated incubation mixture conditions (including anti-BSA antibody, PCMT and AdoMet) were slightly higher than in the unmethylated incubation mixture condition excluding PCMT

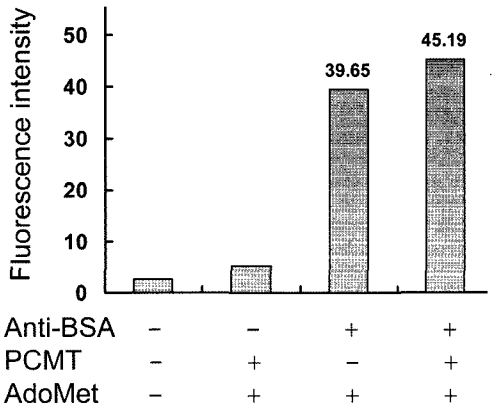

Fig. 2. Effect of carboxylmethylation on the change in hydrophobicity anti-BSA antibody assessed by fluorescence spectrophotometry. Unless otherwise indicated, all determinations and experiments were performed in a cuvette containing a fixed concentration of anti-BSA antibodies (5 μM) and Bis-ANS (1 μM) using 3 mL of a 20 mM sodium phosphate buffer (pH 8.0). The fluorescence spectrophotometer were operated in scan mode and performed using a 5 nm band-pass for excitation, 10 nm for emission, 240 nm/min for scan speed and 0.5 sec for response. Data are the mean of four separate experiments.

from 39.65 to 45.19 (11.4%). This indicates that the total hydrophobicity was increased by carboxylmethylation, even though there was no gross change.

Effect of carboxylmethylation of anti-BSA antibody on antigen-binding activity

The degree of antigen-binding activity was examined by ELISA In order to determine the effect of carboxylmethylation of the anti-BSA antibody on its functional role. Fig. 3 shows that there was no dramatic alteration in the antigen-antibody reaction but there was a statistically significant difference ($p = 0.0217$) between the carboxylmethylated (anti-BSA antibody + PCMT + AdoMet) and the uncarboxylmethylated (anti-BSA antibody + AdoMet) conditions of the anti-BSA antibody. In particular, the carboxylmethylated anti-BSA antibody had an up to 25% higher binding capacity than the uncarboxylmethylated anti-BSA antibody, suggesting that protein carboxylemthylation can affect the principle role of immunoglobulin.

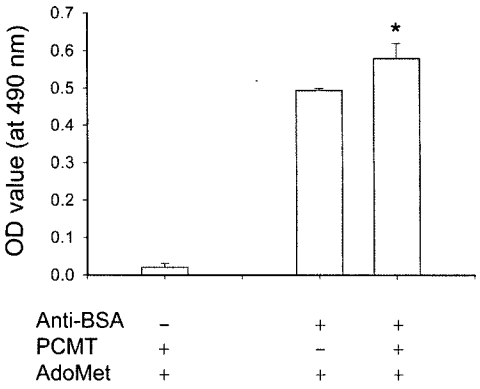

Fig. 3. Effect of carboxylmethylation on the antigen-binding reactivity of the anti-BSA antibodies assessed by ELISA. The reaction is allowed to proceed for 15 minutes under various conditions. The anti-BSA antibody (125 μM) and the secondary antibody (goat anti-rabbit IgG antibody labeled peroxidase) (1 : 5000) were used to prepare the carboxylmethylation reaction in the presence or absence of PCMT and AdoMet, as described in Materials and methods. The absorbance was measured at 490 nm. The results represent the data from two sets of means of four separated experiments. * $P < 0.05$.

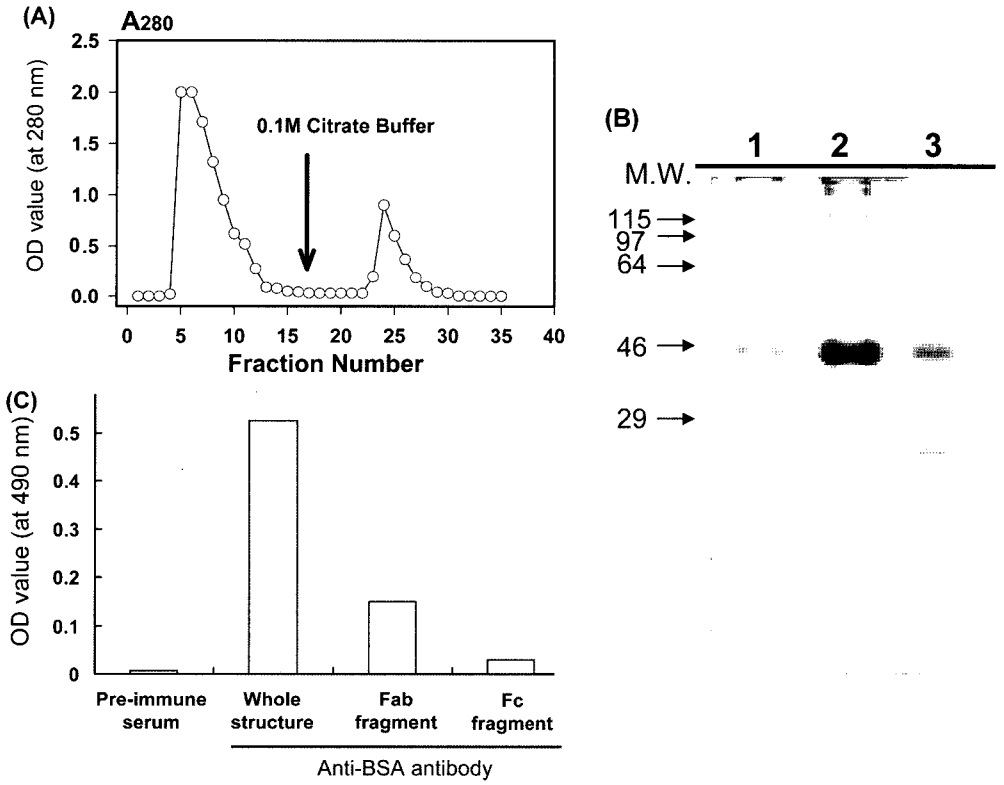

Fig. 4. Preparation of the Fab and Fc fragments. (A) Affinity chromatography of the digested anti-BSA antibody on an antigen Sephrose-6B column. The column (0.5×12 cm) was equilibrated and chromatographed with 20 mM phosphate buffer, pH 7.3. The digested anti-BSA antibodies were applied in the same buffer. The unbinding fractions (Fc fractions) were pooled and concentrated by ultrafiltration using an Amicon apparatus. Elution of the adsorbed protein (Fab fragments) was carried out with a 100 mM citrate buffer, pH 2.8. (B) SDS-PAGE of purified Fab and Fc fragments. SDS-PAGE was carried out in 12% acrylamide gels, as described in the materials and method section. The molecular mass standards (β-galactosidase, 115000; Phosphorylase B, 97400; Bovine albumin, 64000; Egg albumin, 46000; carbonic anhydrase, 29000) were used. Lane 1: partially digested anti-BSA antibody; Lane 2: Fc fraction; Lane 3: Fab fraction (C) ELISA of Fab and Fc fragments. ELISA was carried out as reported in the method section. Samples (1 μM each) and the secondary antibody (goat anti-rabbit IgG antibody labeled peroxidase) (1 : 5000) were used. Data are mean of four separated experiments.

Preparation of Fab and Fc fragments

In order to understand which portion was carboxylmethylated, the anti-BSA antibody was treated with papain and the digested fragments were separated by BSA-affinity chromatography to obtain the Fab and Fc fractions (Fig. 4A). An analysis by SDS-PAGE indicated a 46 kDa fragment of the major band in the purified Fab and Fc fragments (Fig. 4B lanes 2 and 3). ELISA was used to monitor the antigen-binding capacity of the purified Fab and Fc fragments. Fig. 4C shows that the overall binding activity of the anti-BSA antibody was lower in the Fab condition up to 70%, and almost disappeared in the Fc structure.

Fc region is the major carboxylmethylation site in anti-BSA antibody

The methyl accepting capacity of the purified Fab and Fc fragments was carefully evaluated. Interestingly, the

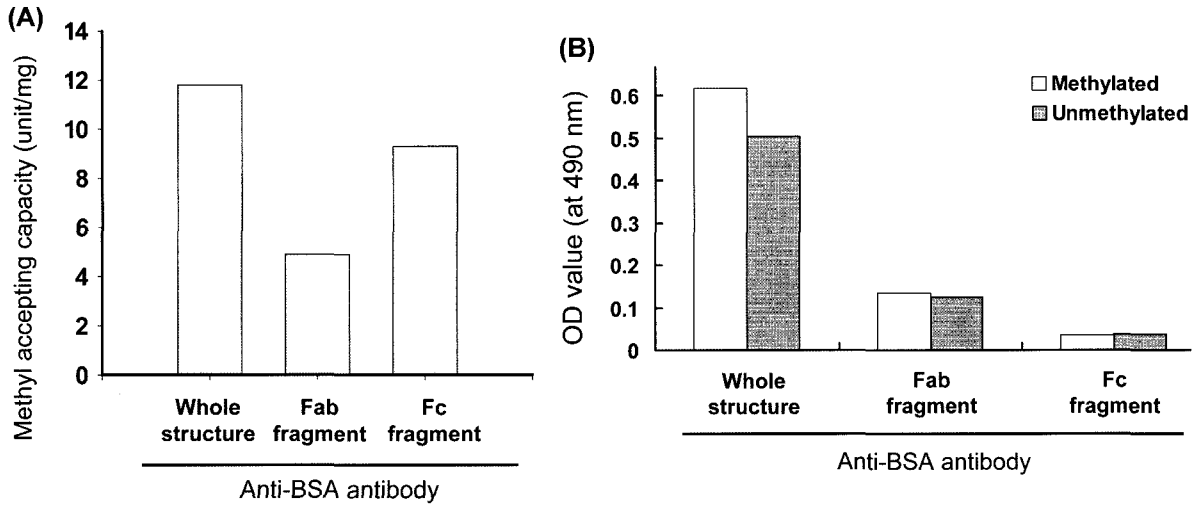

Fig. 5. Carboxylmethylation of the Fab and Fc fragments and their antigen-binding reactivity. (A) Carboxylmethylation of the Fab and Fc fragments. The methyl accepting capacity was measured using the methanol extraction method. One unit of the methyl accepting capacity is defined as the amount of substrate that is catalyzed the transfer of one pmole of methyl-^{14}C groups incorporated to the substrate proteins per minute. (B) Effect of carboxylmethylation on the Fab and Fc fragments assessed by ELISA. Carboxylmethylated or unmethylated samples (125 mM each) were used for ELISA. The data represents the mean of four separate experiments.

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                                                  *
A mouse  IgG1   231  ICTVPEVSSV  FIFPPKPKDV  LTITLTPKVT  CVVVDISKDD  PEVQFSWFVD  DVEVHTAQTQ
  mouse  IgG2a      APNLLGGPSV  FIFPPKIKDV  LMISLSPIVT  CVVVDVSEDD  PDVQISWFVN  NVEVHTAQTQ
  mouse  IgG2b      APNLEGGPSV  FIFPPNIKDV  LMISLTPKVT  CVVVDVSEDD  PDVQISWFVN  NVEVHTAQTQ
  human  IgG1       APELLGGPSV  FLFPPKPKDT  LMISRTPEVT  CVVVDVSHED  PEVKFNWYVD  GVEVHNAKTK
  rabbit IgG        PPELLGGPSV  FIFPPKPKDT  LMISRTPEVT  CVVVDVSQDD  PEVQFTWYIN  NEQVRTARPP
                                                  *
  mouse  IgG1   291  PREEQFNSTF  RSVSELPIMH  QDWLNGKEFK  CRVNSAAFPA  PIEKTISKTK  GRPKAPQVYT
  mouse  IgG2a      THREDYNSTL  RVVSALPIQH  QDWMSGKEFK  CKVNNKDLPA  PIERTISKPK  GSVRAPQVVV
  mouse  Igg2b      THREDYNSTI  RVVSTLPIQH  QDWMSGKEFK  CKVNNKDLPS  PIERTISKIK  GLVRAPQVYI
  human  IgG1       PREEQYNSTY  RVVSVLTVLH  QDWLNGKEYK  CKVSNKSLPA  PIEKTISKAK  GQPREPQVYT
  rabbit IgG        LREQQFNSTI  RVVSTLPITH  QDWLRGKEFK  CKVHNKALPA  PIEKTISKAR  GAPLEPKVYT
                                                  #
  mouse  IgG1   351  IPPPKEQMAK  DKVSLTCMIT  DEFPEDITVE  WQWNGQPAEN  YKNTQPIMDT  DGSYFVYSKL
  mouse  IgG2a      LPPPEEEMTK  KQVTLTCMVT  DFMPEDIYVE  WTNNGKTELN  YKNTEPVLDS  DGSYFMYSKL
  mouse  IgG2b      LPPPAEQLSR  KDVSLTCLVV  GFNPGDISVE  WTSNGHTEEN  YKDTAPVLDS  DGSYFIYSKL
  human  IgG1       LPPSREEMTK  NQVSLTCLVK  GFYPSDIAVE  WESNGQPENN  YKTTPPVLDS  DGSFFLYSKL
  rabbit IgG        MGPPREELSS  RSVSLTCMIN  GFYPSDISVE  WEKNGKAEDN  YKTTPVALDS  DGSYFLYNKL
                                                  #
  mouse  IgG1   411  NVQKSNWEAG  NTFTCSVLHE  GLHNHHTEKS  LSHSPGK
  mouse  IgG2a      RVEKKNWVER  NSYSCSVVHE  GLHNHHTTKS  FSRTPGK
  mouse  IgG2b      NMKTSKWEKT  DSFSCNVRSE  GLKNYYLKKT  ISRSPGK
  human  IgG1       TVDKSRWQQG  NVFSCSVMHE  ALHNHYTQKS  LSLSPGK
  rabbit IgG        SVPTSEWQRG  DVFTCSVMHE  ALHNHYTQKS  ISRSPGK
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Fig. 6. Putative carboxylmethylation sites in Fc region of the immunoglobulin heavy chain. The residues that were reported to be important for an interaction with protein A are indicated by * and the nighboring domains of the interaction core are marked by #.

carboxylmethylation of immunoglobulin was mainly observed in the Fc region. Fig. 5A shows that 80% of the total methyl accepting capacity was observed in the Fc portion, whereas 40% were observed in the Fab portion, suggesting that the Fc fragment is the major carboxylmethylation site in immunoglobulin. However, these carboxylation patterns did not affect the antigen-binding reactivity of the carboxylmethylated Fab fragment. On the other hand, a change was observed in the whole structure of the carboxylmethylated anti-BSA antibody (Fig. 5B), suggesting that the carboxylmethylation of Fc may participate in a total structural change.

Putative carboxylmethylation site in Fc region of anti-BSA antibody

The amino acid sequences of mouse IgG1, 2a, 2b, human IgG1 and rabbit IgG heavy chain constant region were aligned in order to analyze the putative carboxylmethylation site [aspartic acid (D) and glutamic acid (E)] in the Fc portion (Fig. 6). Interestingly, 7 aspartic acids and 5 glutamic acids were found to be conserved between humans, mice and rabbits. In particular, two of each amino acid were positioned at sites that are important for an interaction with protein A, and the neighboring domains of the interaction core (Nagaoka and Akaike, 2003). This suggests that carboxylmethylation may be able to change the overall Fc structure and function of immunoglobulin.

Stability of carboxylmethyl ester in carboxylmethylated anti-BSA antibody

The stability of carboxylmethylation was examined under different pH conditions (Fig. 7A) and incubation times (Fig. 7B). The results showed that a low pH (6.0) upregulated the methyl accepting capacity and a long incubation time (up to 90 min) allowed spontaneous demethylation.

DISCUSSION

γ-globulin is an excellent substrate for protein carboxyl methylation by PCMT, and is ideally suited for structure-function studies on account of its multiple biological activity (Edger and Hope, 1976). Hence, γ-globulin would appear to be a good candidate for examining what methylation really means in terms of protein function. Therefore, in this study, immunoglobulin (anti-BSA antibody), a specific form of γ-globulin that plays a central role in humoral immune response, binding antigens and inactivating them or triggering the inflammatory responses (Presta, 2002), was chosen to examine the possibility that protein carboxylmethylation can participate in immunological events. As expected, the anti-BSA antibody was shown to have similar carboxylmethylation potency to γ-globulin, as a natural substrate for PCMT (Table I), which was demonstrated through its change in hydrophobicity (Fig. 2) because carboxylmethylation itself is a phenomenon that can replace an anionic charge with neutral one. The methyl accepting capacity of the anti-BSA antibody and γ-globulin was higher than that of pepsin, cytochrome C and gelatin, which are also known to be non-specific substrate proteins, highlighting the superior carboxylmethylation potency of immunoglobulin. For this reason, the post-translational modification process would be another important reaction that regulates the roles of immunoglobulins, functionally or non-functionally, in a similar manner to glycosylation (Wright and Morrison, 1997; Renfrow *et al.*, 2005) and myristoylation (Pillai and Baltimore, 1987).

The change in polarity as a result of carboxylmethylation causes a conformational change that importantly regulates the molecular function (Paik and Kim, 1992). Therefore, in order to clarify the involvement of protein carboxylmethylation in the immune responses, it is impor-

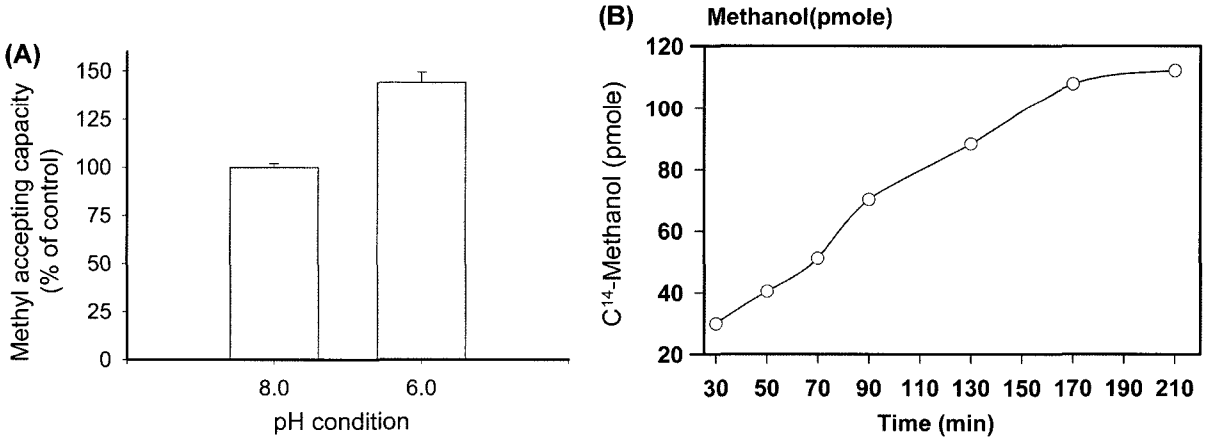

Fig. 7. Reversible demethylation of the carboxylmethylated anti-BSA antibody. (A) Effect of the pH change indicated. (B) Effect of the incubation time indicated. The degree of demethylation was measured using the modified methanol extraction method. The data represents the mean of four separate experiments.

tant to know if the carboxylmethylation of immunoglobulin alters its molecular function. To accomplish this, this study tested the following two points: the outcome of carboxylmethylation of the anti-BSA antibody and the portion of immunoglobulin carboxylmethylated. According to the antigen-binding assay by ELISA, the carboxylmethylated anti-BSA antibody showed significantly higher binding ability to antigen BSA ($p \approx 0.0217$), although the effect was not extreme (Fig. 3). Interestingly, a higher methyl accepting capacity was found in the Fc fragments rather than in the Fab fragments, even though Fab had 40% of the whole body methyl accepting capacity (Fig. 5A). In particular, carboxylmethylation in the Fab fragment did not affect the binding capacity of FAB to BSA. However, carboxylmethylation of the whole structure did affect its binging capacity. This suggests that the carboxylmethylation of Fc may be more effective in regulating the antigen binding capacity of the whole immunoglobulin structure. Indeed, the regulating effect of the Fc portion on the antigen binding capacity was also demonstrated by the antibody function assay and flow cytometric analysis. Therefore, a comparison of the efficacy of whole CD98 antibody to F(ab')2, which is a form where the Fc portion has been removed, on the induction of cell adhesion and binding ability of cell surface antigen showed that F(ab')2 was clearly less effective, up to 15 to 20% less effective (Cho *et al.*, 2001). Similarly, the total binding effect of the carboxylmethylated antibody to BSA was also up to 20% higher. It was reported that the antigen-binding reactivity is mainly determined by the hydrogen bond strength, not hydrophobicity (James and Tawfik, 2003). However, the regulating effect of carboxylmethylation on the function of immunoglobulin may not be critical. Therefore, a Fc region carboxylmethylation-mediated conformational change may not alter the opening local antigen-binding site but give some overall torsion to enhance the antigen-binding opportunity.

The amino acid sequence of the immunoglobulin G heavy chain had a high homology regardless of the species (Nagaoka and Akaike, 2003). In particular, the amino acid sequence aliment of mouse IgG1, 2a, 2b, human IgG1 and rabbit IgG heavy chain constant region (Fig. 6) suggests that the Fc portion may be important in performing the various biological functions. Indeed, the Fc fragments may mediate the effector functions, such as half-life control, complement fixation and the transfer of antibodies across the placental membrane (Reid, 1983; Wright and Morrison, 1997). Although the involvement of carboxylmethylation of immunogluboin Fc region in these effector functions could not be conclusively demonstrated, several facts suggest that 1) carboxylmethylation enhances the hydrophobicity of immunoglobulin (Fig. 2), 2) seven aspartic acids and five glutamic acids, as putative carboxylmethylation sites, were also found to be conserved in the Fc portion between different species (Fig. 6), 3) carboxylmethyl esters are labile at higher pH (Fig. 7B) and 4) there is a spontaneous demethylation process that occurs in a time-dependent manner (Fig. 7B), which indicates its potential participation in a reversible manner. Therefore, the up-regulation of hydrophobicity may be involved in the antibody-secretion pathway (*via* secretary vesicle with low pH), as shown in the case of myrstoylation (Pillai and Baltimore, 1987). In addition, the carboxylmethylation of immunoglobulin may play a role in simply repairing aged immunoglobulin. This is because the immunoglobulin G family has a relatively long half life, up to 25 days (Goldsby *et al.*, 2003), where it can be damaged by environmental stress. This may be because the most important role of PCMT is to convert atypical amino acids such as L-isoaspartyl residue to normal amino acids (Ingrosso *et al.*, 2000; Farrar and Clarke, 2002; Clarke, 2003) and repairing an aberrant antibody is one of the important biochemical reactions since a structurally abnormal antibody including altered carbohydrate structures can be involved in several disease states (Wright and Morrison, 1997; Alexander *et al.*, 1982). The importance of repairing aged proteins in the body's immune function was recently demonstrated using a PCMT knockout strategy. Under PCMT-/- condition, the bone marrow developed high titers of anti-DNA autoantibodies and the kidney was immunopathologically damaged, as is observed in systemic lupus erythematosus (Doyle *et al.*, 2003). Furthermore, an analysis of natural substrates for PCMT demonstrated that protein carboxylmethylation in the spleen plays a key role in repairing aged proteins (Cho *et al.*, 2004).

In conclusion, the anti-BSA antibody can be carboxylmethylated by spleen PCMT to a level similar to γ-globulin, which is a well-known substrate for PCMT. This carboxylmethylation leads to increased hydrophobicity of the anti-BSA antibody and enhanced the antigen-binding activity. In particular, the Fc region showed a higher methyl accepting capacity, up to 80% of whole structure level. According to the amino acid sequence alignment, 7 aspartic acids and 5 glutamic acids, as potential carboxylmethylation sites, were found to be conserved between species in the Fc portion. The carboxylmethylation of the anti-BSA antibody was reversibly demethylated at higher pH and long incubation times. Therefore, protein carboxylmethylation may reversibly regulate the antibody-mediated immunological events *via* a conformational change in the Fc region. Future projects will include more studies to examine the clear involvement of protein carboxylmethylation in the Fc region-mediated effector functions, such as half-life control, complement fixation and the transfer of antibodies across the placental membrane. Furthermore,

it is unclear if the carboxylmethylation of immunoglobulin affects the combination efficiency of the Fc and Fab portions. Therefore, a more detailed study using advanced detection techniques such as mass spectrometry will be needed.

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