Acrolein, a representative carcinogenic aldehyde, that could be ubiquitously generated in biological systems under oxidative stress shows facile reactivity with a nucleophile such as a protein. In this study, to gain a better understanding of the molecular basis of acrolein modification of protein, we characterized the acrolein modification of a model peptide (the oxidized B chain of insulin) by electrospray ionization-liquid chromatography/mass spectrometry method and established a novel acrolein-lysine condensation reaction. In addition, we found that this condensation adduct represented the major antigenic adduct generated in acrolein-modified protein. To identify the modification site and structures of adducts generated in the acrolein-modified insulin B chain, both the acrolein-pretreated and untreated peptides were digested with V8 protease and the resulting peptides were subjected to electrospray ionization-liquid chromatography/mass spectrometry. This technique identified nine peptides, which contained the acrolein adducts at Lys-29 and the N terminus, and revealed that the reaction of the insulin B chain with acrolein gave multiple adducts, including an unknown adduct containing two molecules of acrolein per lysine. To identify this adduct, we incubated N-acetyllysine with acrolein and isolated a product having the same molecular mass as the unknown acrolein-lysine adduct. On the basis of the chemical and spectroscopic evidence, the adduct was determined to be a novel pyridinium-type lysine adduct, N(6)-(3-methylpyridinium)lysine (MP-lysine). The formation of MP-lysine was confirmed by amino acid analysis of proteins treated with acrolein. More notably, this condensation adduct appeared to be an intrinsic epitope of a monoclonal antibody 5F6 that had been raised against acrolein-modified protein.

Several lines of evidence indicate that the oxidative modification of protein and the subsequent accumulation of the modified proteins have been found in cells during aging and oxidative stress and in various pathological states including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis (1–4). The important agents that give rise to the modification of a protein may be represented by reactive aldehydic intermediates such as ketoaldehydes, 2-alkenals and 4-hydroxy-2-alkenals (3, 5, 6). These reactive aldehydes are considered important mediators of cell damage because of their ability to covalently modify biomolecules, which can disrupt important cellular functions and can cause mutations (5). Furthermore, the adduction of aldehydes to apolipoprotein B in low density lipoproteins has been strongly implicated in the mechanism by which low density lipoproteins is converted to an atherogenic form that is taken up by macrophages, leading to the formation of foam cells (7, 8).

Acrolein, an unpleasant and troublesome byproduct of overheated organic matter, occurs as a ubiquitous pollutant in the environment, e.g. the incomplete combustion of plastic materials, cigarette smoking, and overheating frying oils (9). Acrolein is also formed endogenously through oxidation reactions such as lipid peroxidation, polyamine oxidation by amine oxidase, and myeloperoxidase-catalyzed amino acid oxidation (5, 10). A number of reports have appeared describing the damaging effects of acrolein on the tracheal ciliary movement (11) and the pulmonary wall (12). It has also been shown that acrolein causes chromosomal aberrations, sister chromatid exchanges, and point mutations and reduces the colony-forming efficiency of mammalian cells (13). Moreover, acrolein has been suggested to initiate bladder cancer in rats under certain conditions (14).

Among all of the a,β-unsaturated aldehydes, acrolein is by far the strongest electrophile and therefore shows the highest reactivity with nucleophiles such as proteins (5). Acrolein can display different reactivities toward nucleophilic groups present in a peptide sequence by either reacting with the electrophilic double bond or giving rise to nucleophilic addition on the carbonyl group to form a Schiff base. The former reaction leads to the formation of a simple propanal derivative of amino acids or acrolein-amino acid condensation adduct with a 3-formyl-3,4-dehydroperiperoxy moiety (15). In our previous study (16), to verify the presence of protein-bound acrolein in vivo, we obtained a murine monoclonal antibody 5F6 (mAb5F6) that clearly distinguished the acrolein-modified protein from the native protein. Immunohistochemical analysis of atherosclerotic lesions under oxidative stress shows facile reactivity with a nucleophile such as a protein.
rotic lesions from a human aorta revealed that an acrolein-derived epitope recognized by the antibody indeed constitutes the lesions in which intense positivity is associated primarily with macrophage-derived foam cells (16). In addition, Calingasan et al. (17) have observed a strong immunoreactivity with mAb5F6 in the paired helical filament 1-labeled neurofibrillary tangles of Alzheimer’s disease cases. Furthermore, utilizing an immunoassay system (18), the levels of protein-bound acrolein in human plasma have been recently determined, demonstrating that the extracellular concentrations of protein-bound acrolein in vivo could reach submillimolar ranges (19–22). Based on the observations that (i) the incubation of HPLC-purified acrolein predominantly generated N\(^{-}\)-acetyl-FDP-lysine, and (ii) the binding of the antibody to the acrolein-modified protein was significantly inhibited by the authentic FDP-lysine and, (iii) preadsorption of the antibody by the authentic FDP-lysine totally abolished the immunostainings in the lesions, we tentatively identified FDP-lysine as the major epitope of mAb5F6 (16). However, the nature of the adduct recognized by the antibody remains to be uncertain.

In this study, to gain a better understanding of the molecular basis of the acrolein modification of protein, we extensively analyzed the acrolein-treated insulin B chain by electrospray ionization-liquid chromatography/mass spectrometry (ESI-LC/MS) and ESI-LC/MS/MS. In addition, during the course of this study, we identified a novel pyridinium-type lysine adduct, N\(^{-}\)-3-methylpyridinium)lysine (MP-lysine), and found that this condensation adduct represented a major epitope of a monoclonal antibody 5F6 that had been raised against the acrolein-modified protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—The oxidized B chain of insulin, N\(^{-}\)-acetyl-L-lysine, acrolein, and bovine serum albumin (BSA) were obtained from Sigma. *Staphylococcus aureus* V8 protease (endoprotease Glu-C) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**General Procedures**—NMR spectra were recorded using a Bruker AMX400 (400 MHz) instrument. Ultraviolet absorption spectra were measured with a Hitachi U-Best-50 spectrophotometer.

**Reaction of N\(^{-}\)-Acetyllysine with Acrolein**—The reaction mixture contained 100 mM N\(^{-}\)-acetyllysine and 100 mM acrolein in 50 mM sodium phosphate buffer (pH 7.2). After incubation for 24 h at 37°C, the reaction mixture was analyzed with a reverse-phase HPLC system (SHISEIDO Co. Ltd., Tokyo, Japan) using a Capcell Pak C18 column (SHISEIDO Co., Ltd.). The samples were eluted with a linear gradient of 0.1% acetic acid in water (solvent A)-acetonitrile (solvent B) at a flow rate of 0.2 ml/min, and the column temperature was controlled at 40°C. The elution profile was monitored by absorbance at 215 nm.

**ESI-LCMS/MS Analyses**—The ESI-LCMS and ESI-LCMS/MS analyses were performed on an LCQ Ion trap mass spectrometer (Thermo Electron Co.) equipped with an electrospray ion source. The electrospray system employed a 5-kV spray voltage and a capillary temperature at 280°C. Spectra were acquired in the positive ion mode with a scan range from m/z 200 to 2000. Collision-induced dissociation experiments in the positive ion mode were performed by setting the relative collision energy at 31% using helium as the collision gas.

**RESULTS**

**Enzyme-linked Immunosorbent Assay (ELISA)**—A coating antigen was prepared by incubating 1 mg of BSA with 2 mM acrolein in 1 ml of 50 mM sodium phosphate buffer (pH 7.2) for 2 h at 37°C. A 100-µl aliquot of the antigen solution was added to each well of a 96-well microtiter plate and incubated for 20 h at 4°C. The antigen solution was then removed, and the plate was washed with Tris-buffered saline (TBS) containing 1% Tween 20 (TBS/Tween). Each well was incubated with 200 µl of 1% BSA in TBS/Tween for 30 min at 37°C in a moist chamber to block the unreacted plastic surface. The plate was then washed once with TBS/Tween. A 100-µl aliquot of competitor/antibody mixtures containing antibody at 25 ng/ml and variable concentrations of the competitor was added to each well and incubated for 1 h at 37°C. After discarding the supernatant and washing three times with TBS/Tween, 100 µl of a 5 × 10\(^{-9}\) M solution of goat anti-rabbit IgG conjugated to horseradish peroxidase in TBS/Tween was added after incubation for 1 h at 37°C. The supernatant was discarded and the plates were washed three times with TBS/Tween. Enzyme-linked antibody bound to the well was revealed by adding 100 µl of 0.1 M citrate/phosphate buffer (pH 5.0) containing 0.003% hydrogen peroxide. The reaction was terminated by the addition of 50 µl of 2 M sulfuric acid, and absorbance at 492 nm was read on a micro-ELISA plate reader. Results were expressed as the ratio B/Bo, where B = [absorbance in the presence of competitor] - [absorbance in the absence of competitor] - [background absorbance].

**ESI-LCMS/MS Analysis of Acrolein-modified Insulin B Chain**—To gain a better understanding of the molecular basis of the acrolein modification of protein, oxidized insulin B chain containing four possible target amino acid residues (His-5, His-10, Lys-29, and N-terminal phenylalanine) was chosen as the model peptide. The native and acrolein-treated insulin B chain were digested with V8 protease and then analyzed by ESI-LCMS/MS. V8 protease digestion of insulin B chain theoretically generates three fragments, namely FVNQH’LC*GSHLVE (A), ALYLVC*GE (B), and RGGYTPPKA (C). As shown in Fig. 1 (chromatograms A), limited proteolysis of native insulin B chain with V8 protease indeed gave three fragments, a, b, and c. These unmodified fragments showed the [M + H\(^{+}\)] at m/z 1086.7 (fragment a), 1530.7 (fragment b), and 915.3 (fragment c) (Fig. 2) that were consistent with their calculated molecular mass of [M + H\(^{+}\)] = 1086.6 (B3), 1530.7 (B1), and 915.4 (B2), respectively. On the other hand, reaction of the peptide with acrolein (1–10 mM) followed by V8 protease digestion resulted in the formation of a complex mixture of different species (fragments d, e, f, g, h, and i) whose relative abundance slightly changed with the concentrations of acrolein (Fig. 1, chromatograms B–E). On the basis of their mass value, it was presumed that the fragments d, e, f, and g were originated from the modification of the sequence B1 and the frag-
ments h and i were originated from the modification of the sequence B3 (Fig. 2). Acrolein was anticipated to form three different types of adducts, namely the aldimine-, propanal-, and FDP-type adducts (Fig. 3), resulting in the increments of 38, 56, and 94 Da, respectively, in the mass value of the peptide. Relative to the calculated masses of the unmodified fragments, two fragments (d and e) were shown to have a mass increase of 38 Da corresponding to a Schiff base adduct (Fig. 2). We also detected two fragments f and h, which showed an increased mass of 94 Da, and two fragments g and i, which had an increased mass of 76 Da (Fig. 2).

Presence of Unknown Dimeric Acrolein Species in the Acrolein-modified Insulin B Chain—Based on the fact that B3 contains only one possible target amino acid (Lys-29), it was suggested that the mass increments of 94 and 76 Da in fragments h and i, respectively, were originated from the modification of the lysine residue with two acrolein molecules. However, such acrolein modification of lysine, corresponding to the mass increment of 76 Da, is unknown. In addition, it is also unclear whether fragments f and g, which had the increased mass of 76 and 94 Da, represent singly or doubly modified peptides with two acrolein molecules. Hence, to further characterize the acrolein modification of the insulin B chain, both unmodified fragments (a, b, and c) and acrolein-modified fragments (d, e, f, g, h, and i) were analyzed by ESI-LC/MS/MS without additional chromatography. The MS/MS analysis of the [M + H]+ at m/z 1086.7 from fragment a, the [M + H]+ at m/z 1530.7 from fragment b, and the [M + H]+ at m/z 915.3 from fragment c confirmed the identity of the sequences and their lack of modifications (Supplemental Fig. S1, panels a–c). Table 1 summarizes the MS/MS analysis of the V8 protease-digested acrolein-modified insulin B chain. The presence of an adduct containing the mass addition of 38 Da on Phe (N terminus) was confirmed in fragments d and e (Supplemental Fig. S1, panels d and e). The MS/MS analysis of fragments f and h confirmed the identity of these peptides and the presence of an FDP-type adduct containing the mass addition of 94 Da on Phe (N terminus) and Lys-29, respectively (Supplemental Fig. S1, panels f and h). The MS/MS analysis of fragments g and i revealed the occurrence of a single modifying group with a mass addition of 76 Da and identified the acrolein modification sites to be Phe (N terminus) and Lys-29, respectively (Supplemental Fig. S1, panels g and i). The appearance of such mass increment suggested the presence of some unknown dimeric acrolein species in the peptides. In addition, no shift of the molecular mass of the peptides was observed after treatment with NaCNBH3 (data not shown). This observation suggests that the unknown adduct may not have aldehyde and aldime bonds.

Identification of a Novel Acrolein-Lysine Adduct—To characterize the putative acrolein adduction to primary amine corresponding to the mass increment of 76 Da, Nα-acetylatedlysine was incubated with an equimolar concentration of acrolein in 0.1 M sodium phosphate buffer (pH 7.2) for 24 h and the products were analyzed by LC/MS. As shown in Fig. 4, the LC/MS analysis with selected ion monitoring detected one product (product 2), which showed a 76-De increase in the mass value of the unmodified lysine derivative and gave the [M + H]+ peak at m/z 264.9. To characterize the chemical structure of the product, isolation by HPLC on the reverse-phase column was carried out. After purification, the structure of the product was characterized by NMR analysis. Compared with the 13C NMR spectra between Nα-acetylatedlysine and the product, eight signals (δC 14.8, 20.0, 26.0, 130.7, 143.8, 144.5, 145.9, and 153.3) remained in the 13C NMR spectrum of the product, which seemed to originate from two molecules of acrolein in the reaction with Nα-amine of Nα-acetylatedlysine (Supplemental Fig. S2). The five signals (δC 130.7, 143.8, 144.5, 145.9, and 153.3) suggested the presence of an aromatic ring. The remaining three signals corresponded to two methyls (δH 1.13/6C 14.8; δH 2.63/6C 26.0) and one methylene (δH 2.65/6C 26.0). In addition, the UV spectrum of product 2 exhibited a λmax, 276 nm. These data suggested that the product was composed of one pyridinium ring and one methyl group. An 1H-detected multiple-bond heteronuclear multiple quantum coherence (HMBC) experiment showed the correlations of the e-CH2 protons and methyl protons (δH 2.63) to δC 153.3 (C-2), but a correlation of the ethyl protons to C-2 was not observed, indicating the presence of the methyl group at the C-2 position. In addition, the HMBC experiment also showed the correlations of the e-CH2 protons to C-6; H-6 showed correlations to CH2 (δC 26.0), C-2, and C-4 (1.29 ppm); and CH3 (δH 1.13) showed correlations to CH3 (δC 26.0) and C-5 (Fig. 5). Based on these characteristics, it was determined that product 2 was a novel acrolein-lysine adduct, Nα-acetyl-Nα-(3-methylpyridinium)lysine (Nα-acetyl-MPlysine).

Formation of MP-Lysine in Acrolein-modified Protein—The reactivity of acrolein to the protein was briefly evaluated by SDS-PAGE analysis in which the expanse and mobility shift of the protein bands were observed (Fig. 6A). To assess the formation of MP-lysine in the acrolein-modified proteins, we first attempted to detect the adduct in the hydrolyzed samples of authentic Nα-acetyl-MP-lysine. Upon acid hydrolysis followed...
by amino acid analysis of N^\text{\textbar}^\text{\textbar}^\text{\textbar}^\text{\textbar}^\text{\textbar}^-\text{acetyl-MP-lysine}, a new peak that was distinguishable from other amino acids was detected (Fig. 6B). In addition, the same peak was detected in the hydrolysate of BSA treated with acrolein. Hence, we characterized the stoichiometry of the loss of lysine residues and the concomitant formation of the acrolein-lysine adducts, FDP-lysine, and MP-lysine in the protein exposed to acrolein. As shown in Fig. 6C, a significant loss of lysine residues was observed when BSA (1 mg/ml) was exposed to 0–10 mM acrolein in 50 mM sodium phosphate buffer (pH 7.2) for 24 h at 37 °C. In addition, the loss of lysine residues was accompanied by the formation of both acrolein-lysine adducts. There was a marked tendency for the FDP-lysine to be more preferentially formed than the MP-lysine at lower concentrations of acrolein. However, even after the formation of the FDP-lysine reached a plateau when the protein was treated with 10 mM acrolein, the formation of MP-lysine continued to increase with further increases in the concentration of acrolein. Upon incubation of BSA with the highest level of acrolein (10 mM) for 24 h at 37 °C, ~43 mol of lysine residues/mol protein was lost (the total number of lysine residues per mol protein obtained by amino acid analysis was 60 molecules). These losses were accompanied by the formation of FDP-lysine (21 mol/mol) and MP-lysine (10 mol/mol), accounting for ~49 and 23% of the lysine residues that had disappeared, respectively (Fig. 6C).

Identification of MP-Lysine as the Major Epitope of a Monoclonal Antibody against Acrolein-modified Protein—To investigate whether acrolein is produced in vivo, we previously raised mAb5F6 against acrolein-modified protein and demonstrated that acrolein was accumulated in the form covalently bound to proteins in vivo (10, 16, 17). In addition, based on the observations that the incubation of N^\text{\textbar}^-\text{acetyllysine} with acrolein predominantly generated FDP-lysine and that the antibody indeed recognized it, we regarded FDP-lysine as the epitope of the antibody (16). However, we found in this study that the reaction of the lysine derivative with acrolein formed not only FDP-lysine but also another condensation product, MP-lysine. Since characterization of the ability of antibodies to recognize
specific molecular targets in their native three-dimensional conformation is critical to the use of these reagents, we sought to identify the intrinsic epitope recognized by mAb5F6. To identify the acrolein-lysine adduct recognized by the antibody, the immunoreactivity with the reaction products of acrolein with N-acetyllysine was characterized. As shown in Fig. 7, the ELISA analysis of HPLC fractions for immunoreactivity with mAb5F6 showed that the antibody had immunoreactivity not only with the FDP-lysine but also with the peak corresponding to MP-lysine. More strikingly, the ratio of immunoreactivities to the yields of these products (FDP-lysine and MP-lysine) suggested that the antibody might be rather specific to MP-lysine. This assumption was confirmed by the competitive ELISA analysis in which the binding of the acrolein-modified protein to mAb5F6 was more significantly inhibited by MP-lysine (Fig. 8). Approximately 50 pmol of MP-lysine/well (100 μl) caused 50% inhibition of antibody binding to the acrolein-modified protein, whereas at least 100-fold higher concentrations of FDP-lysine was necessary for the same inhibition. Thus, MP-lysine appeared to be an intrinsic epitope of mAb5F6.

**DISCUSSION**

Among all of the α,β-unsaturated aldehydes, acrolein exhibits the greatest reactivity with nucleophiles such as proteins (5). Upon reaction with a protein, acrolein selectively reacts with the side chains of the cysteine, histidine, and lysine residues. Of these residues, lysine generates the most stable product. The β-substituted propanals (R-NH-CH₂-CH₂-CHO) and Schiff base cross-links (R-NH-CH₂-CH₂-CH=N-R) had been suggested as the predominant adducts; however, the major adduct formed upon the reaction of acrolein with protein was identified to be FDP-lysine, which requires the attachment of two acrolein molecules to one lysine side chain (15, 16). The formation of a similar FDP-type adduct (dimethyl-FDP-lysine) was also reported in the lysine modification with the acrolein analogue, crotonaldehyde (25). In addition, these FDP-type adducts have been detected in the reaction of other 2-alkenals, such as 2-pentenal and 2-hexenal, with the lysine derivative (25), suggesting that the condensation reaction via the formation of the Michael addition-derived imine derivatives is characteristic of the reaction of 2-alkenals with primary amines. Because the core structure of the FDP-lysine is resistant to the conventional acid hydrolysis of proteins even without reduction by pretreatment with sodium borohydride, the FDP-type adducts of acrolein and crotonaldehyde have been successfully detected in the acrolein-modified proteins (15) and crotonaldehyde-modified proteins (25), respectively. Meanwhile, in contrast to the predominant generation of the FDP-lysine adduct in the acrolein modification of lysine derivatives, the loss of lysine residues in protein upon the incubation of acrolein was only partially recovered by the formation of the adduct. For example, the treatment of BSA with acrolein resulted in the loss of ~50 mol of lysine residues but a gain of only 20 mol of FDP-lysine moieties/mol protein (15). These observations...
strongly suggested that, upon reaction with the lysine residues, acrolein could generate product(s) other than FDP-lysine. Candidate products included the propanal and Schiff base cross-link. Indeed, the incubation of BSA with acrolein formed a faint protein band corresponding to the oligomer (dimer) of BSA (Fig. 6A). Our previous study also provided evidence that FDP-lysine is not a stable end product but a reactive intermediate that covalently binds to thiols (26).

In this study, to identify the modification site and structures of adducts generated in acrolein-modified protein, a model peptide (insulin B chain) treated with acrolein was analyzed by the ESI-LC/MS and ESI-LC/MS/MS methods. Relative to the calculated masses of the unmodified fragments, the presence of several types of adducts including FDP- and aldimine-type adducts was confirmed by these techniques. It was revealed that the aldimine-type adduct containing the mass addition of 38 Da was generated on the N-terminal Phe (Fig. 2). In addition, the MS/MS analysis of fragments f and h confirmed the identity of these peptides and the presence of the FDP-type adduct containing the mass addition of 94 Da on Phe (N terminus) and Lys-29, respectively (Supplemental Fig. S1, panels f and h). It should be noted that a 56-Da increase in the molecular mass of the native peptide, corresponding to the formation of a propanal-type adduct via a nucleophilic addition on the acrolein double bond, was not observed. This is probably because of an interaction between the propanal adduct and the primary amino group of Lys or N-terminal Phe, generating an intramolecular cross-link. Carbene et al. (27) have suggested the formation of an intramolecular aldimine bond involving the terminal amino group and the side chain of histidine residue bridged by an acrolein molecule in the acrolein-modified decapeptide (angiotensin I). The authors have also noted that the formation of the intramolecular aldimine bond should be favored by entropic factors. This proposition may be supported by the fact that acrolein has been widely used as a cross-linking reagent in protein chemistry to link covalently different subunits in oligomeric proteins (28).

On the other hand, the MS/MS analysis of fragments g and i revealed the occurrence of a single modifying group with a mass addition of 76 Da and identified the acrolein modification sites to be Phe (N terminus) and Lys-29, respectively (Supplemental Fig. S1, panels g and i). This observation strongly suggested the presence of an unknown dimeric acrolein species. Because such acrolein modification of lysine corresponding to
A Major Antigenic Acrolein-Lysine Adduct

Fig. 8. Competitive ELISA analysis with the acrolein-lysine adducts. A, chemical structures of competitors used. Upper, Nα-acetyl-FDP-lysine; lower, Nα-acetyl-MP-lysine. B, competitive ELISA. Competitors: ○, Nα-acetyl-FDP-lysine; ●, Nα-acetyl-MP-lysine.

the mass increment of 76 Da was unknown, we attempted to identify this adduct using Nα-acetyllysine. Upon incubation of the lysine derivative with acrolein, we identified the lysine-pyridinium adduct (MP-lysine), which resulted from the Schiff base formation of acrolein with the ε-amino group of lysine, as the novel acrolein-lysine adduct. The formation of MP-lysine may be reasonably explained by the mechanism involving the formation of a Schiff base derivative as the first intermediate (Fig. 9). The Schiff base further reacts with a second acrolein molecule via a Michael addition to generate an imine derivative. The subsequent conversion of this imine derivative to the final product (MP-lysine) obviously requires two oxidation steps and intramolecular cyclization, but its detailed mechanism is not yet clear. MP-lysine was detected as a minor product in the reaction of acrolein with the lysine derivative (Fig. 4), whereas the formation of the pyridinium adducts has been reported to be a dominant pathway for the modification of the primary amine with 2-alkenals such as 2-hexenal and 2-octenal (29–31). The formation of these lysine-pyridinium species in proteins may result in the placement of a fixed positive charge on the ε-amino group. Moreover, in contrast to the fact that the FDP-type adducts are unstable intermediates against nucleophilic addition, the pyridinium adducts are highly stable end products.

By amino acid analysis technique, we have previously established the method for the detection of FDP-lysine generated in acrolein-modified proteins (15). In this study, we found that the MP-lysine adduct could also be clearly separated from FDP-lysine and all of the other normal amino acids in the amino acid analysis technique used (Fig. 6). When BSA (1 mg/ml) was incubated with 10 mM acrolein for 24 h at 37 °C, ~21 mol of FDP-lysine and 10 mol of MP-lysine/mmol protein were detected. The yields of the adducts accounted for ~49 and 23% of the lysine residues that had disappeared (Fig. 6), suggesting that the lysine modification by acrolein may be largely explained by the formation of these condensation adducts. Moreover, it is of interest that, although there was a marked tendency for the FDP-lysine to be more preferentially formed than the MP-lysine at lower concentrations of acrolein, a large excess of aldehyde over the amine was not necessarily needed to ensure the formation of these condensation adducts (Fig. 6). The use of equimolar quantities afforded adducts mainly containing two molecules of acrolein per lysine molecule (Fig. 4). Although this finding suggested at the time that acrolein was inherently biased toward condensation (e.g. aldol) chemistry, our ability to obtain these adducts from equimolar acrolein-lysine reactions indicates that the amine is responsible for stimulating the aldehyde condensations.

Immunologic detection is a powerful tool that can be used to evaluate the presence of a desired target and its subcellular localization. Among the major advantages of this technique over biochemical approaches is the evaluation of small numbers of cells or archival tissues that may otherwise not be subject to analysis. In this study, based on the identification of a novel acrolein-lysine adduct (MP-lysine), we re-examined the specificity of mAb5F6 and found that the antibody recognized MP-lysine far more efficiently than FDP-lysine (Fig. 8). The preferential recognition of the antibody to MP-lysine is probably explained by the structural characteristics in the side chain of these adducts. In contrast to FDP-lysine, MP-lysine contains a more fixed positive charge on the pyridinium side chain, which may represent important immunological epitopes. Indeed, a monoclonal antibody raised against crotonaldehyde-modified proteins recognized a similar pyridinium adduct as the major epitope (26). In addition, Nagai et al. (32) have raised a monoclonal antibody against glyoxaldehyde-modified protein and found that a lysine pyridinium adduct constitutes an epitope of the antibody.

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