Elucidation of Reaction Scheme Describing Malondialdehyde−Acetaldehyde−Protein Adduct Formation

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Malondialdehyde and acetaldehyde react together with proteins and form hybrid protein conjugates designated as MAA adducts, which have been detected in livers of ethanol-fed animals. Our previous studies have shown that MAA adducts are comprised of two distinct products. One adduct is composed of two molecules of malondialdehyde and one molecule of acetaldehyde and was identified as the 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative of an amino group (MDHDC adduct). The other adduct is a 1:1 adduct of malondialdehyde and acetaldehyde and was identified as the 2-formyl-3-(alkylamino)butanal derivative of an amino group (FAAB adduct). In this study, information on the mechanism of MAA adduct formation was obtained, focusing on whether the FAAB adduct serves as a precursor for the MDHDC adduct. Upon the basis of chemical analysis and NMR spectroscopy, two initial reaction steps appear to be a prerequisite for MDHDC formation. One step involves the reaction of one molecule of malondialdehyde and one of acetaldehyde with an amino group of a protein to form the FAAB product, while the other step involves the generation of a malondialdehyde−enamine. It appears that generation of the MDHDC adduct requires the FAAB moiety to be transferred to the nitrogen of the MDA−enamine. For efficient reaction of FAAB with the enamine to take place, additional experiments indicated that these two intermediates likely must be in positions on the protein of close proximity to each other. Further studies showed that the incubation of liver proteins from ethanol-fed rats with MDA resulted in a marked generation of MDHDC adducts, indicating the presence of a pool of FAAB adducts in the liver of ethanol-fed animals. Overall, these findings show that MDHDC−protein adduct formation occurs via the reaction of the FAAB moiety with a malondialdehyde−enamine, and further suggest that a similar mechanism may be operative in vivo in the liver during prolonged ethanol consumption.

Introduction

Ethanol metabolism in the liver results in the formation of acetaldehyde, which can covalently bind to hepatic proteins, and this process has been implicated as playing an important role in the development of alcoholic liver injury (*1*, *2*). There is also considerable evidence indicating that chronic ethanol consumption induces oxidative stress and lipid peroxidation in the liver (*3*, *4*) which in turn generates another reactive aldehyde, malondialdehyde (MDA).[1] Studies from our laboratory have demonstrated that acetaldehyde and MDA can react together with proteins in a synergistic manner and form hybrid protein conjugates that have been designated as MAA adducts (*5*). These adducts have been detected in livers of ethanol-fed rats (*5*), and circulating antibodies against MAA adducts have been identified in ethanol-fed rats (*6*) and in patients with alcohol-induced liver disease (*7*). Further studies have shown that proteins with MAA adducts can serve as immunogens and can alter the function of various cell types in the liver. Adduction of proteins with MDA and acetaldehyde results in a potent immune response, resulting in circulating antibodies against both the carrier protein epitopes, as well as to the MAA adduct epitope (*8*). MAA adducts also appear to possess both pro-inflammatory and profibrogenic properties. These adducts have been shown, in vitro, to increase the secretion of chemokines and to upregulate ICAM expression in hepatic stellate cells (*9*) and to increase the secretion of TNFα and chemokines, to upregulate adhesion molecule expression, and to enhance fibronectin secretion in liver endothelial cells (*10*). These findings suggest that MAA adducts may play a significant role in the development of alcoholic liver injury.

Chemical characterization and structural analysis of MAA−protein adducts is an essential step in clarifying

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[1] Abbreviations: MDA, malondialdehyde; MAA, malondialdehyde-acetaldehyde; FAAB, 2-formyl-3-(alkylamino)butanal; MDHDC, 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde; BSA, bovine serum albumin; PLL, poly-L-lysine; hexyl-MDHDC, 1-hexyl-MDHDC; ethyl-MDHDC, 1-ethyl-MDHDC; hexyl-FAAB, 2-formyl-3-(hexylamino)-butanal; ELISA, enzyme-linked immunosorbent assay.

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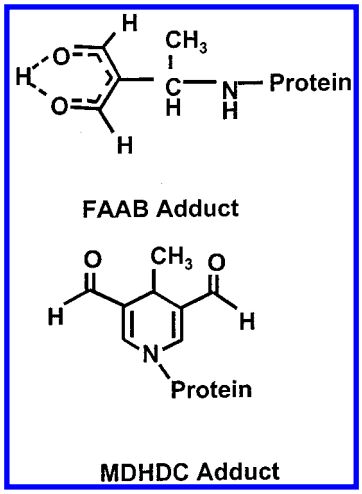

Figure 1. Chemical structures of MAA−protein adducts.

the role of these adducts in ethanol-induced hepatotoxicity. Our previous work showed that two major adducts result from the reaction of MDA and acetaldehyde with proteins, and their structures are depicted in Figure 1 (*11*, *12*). One adduct is a highly fluorescent, cyclic product composed of two molecules of MDA and one molecule of acetaldehyde and has been identified to be a 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative of an amino group (MDHDC adduct). The other adduct is a 1:1 adduct of MDA and acetaldehyde, forming a 2-formyl-3-(alkylamino)butanal derivative of an amino group (FAAB adduct). The mechanism which accounts for the formation of these adducts both in vitro and in vivo is still unclear. Our previous studies showed that when MDA and acetaldehyde are allowed to react with proteins, the FAAB adduct is the prominent adduct at early reaction times, whereas, the formation of the MDHDC adduct is delayed to more prolonged times of reaction (*11*, *12*). These findings suggest that the FAAB adduct may serve as a precursor for the formation of the MDHDC adduct. Therefore, the purpose of the present study was to gain information concerning the mechanism of MAA adduct formation, focusing on whether the FAAB adduct can serve as a precursor for the generation of the MDHDC adduct. Both in vitro and in vivo experiments were conducted for this purpose, and some additional important information concerning reaction sites on proteins undergoing MAA adduction was obtained.

Experimental Procedures

Materials. [1,2-^{14}C]Acetaldehyde (5 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Radiolabeled acetaldehyde was received from the manufacturer frozen as an aqueous solution (1 mCi/mL), thawed, and diluted to 250 μCi/mL with distilled water, rapidly refrozen, and stored at −70 °C. The specific activity of the acetaldehyde was checked as described by Miwa et al. (*13*). Nonradioactive acetaldehyde was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Bovine serum albumin (BSA) (crystallized, lyophilized, and fatty acid free) and poly-L-lysine (PLL) (mol. wt. 16 100) were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

Organic Synthesis. MDA was synthesized as the sodium salt by the method of Grabowski and Autrey (*14*). 1-Hexyl-4-methyl-1,4-dihydro-3,5-pyridinedicarboxaldehyde (hexyl-MDHDC) and 2-formyl-3-(hexylamino)butanal (hexyl-FAAB) were previously synthesized in this laboratory (*11*, *12*). *N*-Ethylaminopropenal has been previously characterized by Makin et al. (*15*) and was synthesized by the method of Nair et al. (*16*). This procedure gives *N*-ethylaminopropenal as a mixture of isomers (E and Z). The ^{1}H NMR signals for each of these compounds are reported below. 1-Ethyl-4-methyl-1,4-dihydro-3,5-pyridinedicarboxaldehyde (ethyl-MDHDC) is a new compound and was synthesized by the same procedure as hexyl-MDHDC (*11*). It was characterized by ^{1}H and ^{13}C NMR, IR spectroscopy, and microanalysis (C, H, N) performed by Atlantic Microlab, Inc. (Norcross, GA). The IR spectrum was recorded using a Nicolet Impact 410 spectrophotometer and melting point was determined on a MelTemp melting point apparatus.

Na-MDA. ^{1}H NMR (D_2O) δ 8.6 (d, 2H), 5.3 (t, 1H). ^{1}H NMR (pD 7.4 buffer) δ 8.7 (s, 2H). In buffer, the α-hydrogen exchanges with the solvent within two minutes.

Hexyl-FAAB. ^{1}H NMR (pD 7.4 buffer) δ 8.6 (s, 2H, −CHO), 4.2 (q, 1H, CH−CH_3), 2.9 (t, 3H, N−CH_2), 1.6 (m, 2H, CH_2−C−N), 1.5 (d, 3 H, CH_3−CH), 1.2−1.4 (m, $(CH_2)_3$), 0.9 (t, 3H, CH_3).

N-Ethylaminopropenal. ^{1}H NMR (pD 7.4 buffer) δ 8.7 (d, 1H, −CHO), 8.6 (d,1H, −CHO), 7.6 (m, 1H and 1H, =CH−N), 5.4 (m, 1H and 1H, CH=C), 3.3 (q, 2 H, N−CH_2), 3.2 (q, 2H,N−CH_2), 1.3 (t, 3H, CH_3), 1.2 (t, 3H, CH_3). These signals are for the mixture of E and Z isomers.

Ethyl-MDHDC. Column chromatography (R_f = 0.56, 1:9 methanol:chloroform) followed by recrystallization from methanol afforded the product as yellow needles: mp 154−155 °C; IR (KBr) 3031, 2963, 1635, 1567, 715 cm^{-1};^{1}H NMR (pD 7.4 buffer) δ 9.2 (s, 2H, −CHO), 7.3 (s, 2H, C=CH), 3.8 (q, 1H, CH−CH_3), 3.6 (q, 2H, N−CH_2), 1.3 (t, 3H, CH_3−CH_2), 1.0 (d, 3H, CH_3−CH); ^{13}C NMR (pD 7.4 buffer) δ 193.0, 150.7, 123.3, 50.2, 22.3, 21.6, 14.4. Anal. calcd for $C_{10}H_{13}NO_2$: C, 67.02; H, 7.31; N, 7.82. Found: C, 66.87; H, 7.21; N, 7.74.

Hexyl-MDHDC. ^{1}H NMR (pD 7.4 buffer) δ 9.2 (s, 2H, −CHO), 7.3 (s, 2H, C=CH), 3.7 (q, 1H, CH−CH_3), 3.6 (t, 2H, N−CH_2), 1.2−1.8 (m, 8 H, $(CH_2)_4$), 1.0 (d, 3H,CH_3−CH), 0.9 (t, 3H, CH_3).

Preparation of FAAB-BSA and FAAB-PLL. A solution of BSA (10 mg/mL) was incubated with 20 mM acetaldehyde and 2 mM MDA, and a solution of PLL (10 mg/mL) was incubated with 10 mM acetaldehyde and 1 mM MDA. Incubations were conducted in phosphate buffer (0.1 M, pH 8.2) at 15 °C for 24 h in polypropylene vessels that were sealed to minimize losses due to volatility. At the end of incubation, the reaction mixtures were exhaustively dialyzed against phosphate buffer (0.1 M, pH 7.4) for 24 h at 4 °C. These reaction conditions generated MAA adducts of BSA and PLL where 99% or greater of the total adducts formed was the FAAB adduct and less than 1% was the MDHDC adduct.

Preparation of MAA Adducts (consisting of a mixture of FAAB and MDHDC adducts) with BSA and PLL. Solutions of BSA (1 mg/mL) and PLL (1 mg/mL) were incubated with 1 mM acetaldehyde and 1 mM MDA in phosphate buffer (0.1 M, pH 7.4) at 37 °C for 3 days. At the end of incubation, the reaction mixtures were exhaustively dialyzed against phosphate buffer (0.1 M, pH 7.4) for 24 h at 4 °C. These reaction conditions yielded MAA adducts composed of 60% FAAB adduct and 40% MDHDC adduct for both BSA and PLL.

Preparation of MDA Schiff Bases of BSA. BSA (1 mg/mL) was incubated with 1 mM MDA in phosphate buffer (0.1 M, pH 7.4) at 37 °C for 24 h. At the end of incubation, the reaction mixture was dialyzed against phosphate buffer for 2 h at 4 °C to get rid of the free, unreacted MDA. This procedure has been shown to yield Schiff base adducts of MDA with BSA, particularly the mono Schiff base derivative, aminopropenal (*17*, *18*).

Quantification of MDHDC and FAAB Adducts. Total MAA adducts were quantified by a [^{14}C]acetaldehyde binding assay as previously described (*5*, *19*). This assay measures the nanomoles of stably bound acetaldehyde per milligram of protein or polypeptide, and based on the known structures of MAA adducts which contain 1 mol of acetaldehyde per mol of MAA derivative, this assay can be used to quantify total MAA adducts on a protein. MDHDC adducts were determined directly by a

fluorescence assay as previously described (*5*, *11*). Fluorescence measurements (excitation max 398 nm and emission max at 460 nm) were obtained on postdialysis samples using a Perkin-Elmer (Norwalk, CT) LS-5B spectrophotofluorometer attached to a Perkin-Elmer GP-100 graphics printer. The data were expressed as nanomoles of MDHDC adduct per milligram of protein or polypeptide, using hexyl-MDHDC as a standard. The quantity of FAAB adducts on a protein or polypeptide were estimated by subtracting the amounts of MDHDC adducts from total MAA adducts. The suitability of this method is based on our previously reported finding that MAA adducts are comprised almost entirely of the FAAB and MDHDC adducts (*12*), and this finding was again confirmed by applying ^{13}C NMR spectra analyses of the variously generated MAA adducts used in this study as previously described (*12*).

NMR Spectroscopy. All ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA 300 spectrometer, and the solvent was 0.5 M phosphate buffer in D_2O (pD 7.4). For ^{1}H NMR, the chemical shifts (δ) are relative to the HOD signal ($\delta = 4.81$ ppm). For ^{13}C NMR, the chemical shifts are relative to 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt ($\delta = -2.58$ ppm). Each ^{1}H NMR reaction consisted of adding 0.030 mmol of each reactant to 0.6 mL of D_2O buffer, and acquiring an initial ^{1}H NMR 16-scan spectrum. The NMR tube was then placed in a 37 °C oil bath and removed at various time intervals and 16-scan spectra acquired.

Analysis of MDHDC−Protein Adducts in Livers of Ethanol-Fed and Control Rats. Male Wistar rats (150−160 g) were pair-fed the Lieber-DeCarli ethanol-containing diet and the control liquid diets (*20*) for up to 11 weeks as previously reported (*21*). Liver homogenates from these animals were prepared in Hepes-buffered sucrose (pH 7.4). In some cases, the livers were perfused as previously described (*22*) in the presence or absence of MDA prior to the preparation of liver homogenates. Relative levels of MDHDC-liver protein adducts were determined by a direct ELISA as previously described in detail (*5*, *6*). An affinity-purified antibody that specifically recognizes MDHDC epitopes on proteins (*11*) was used to assay for MDHDC adducts on liver proteins. Optical density at 405 nm was measured by a Dynatech Micro ELISA Reader MR700 (Dynatech, Chantilly, VA), and increasing optical density readings were reflective of increasing levels of MDHDC adducts.

Results

Reaction of FAAB−Protein Adducts with MDA. When a 10-fold molar excess of acetaldehyde relative to MDA was incubated with BSA at pH 8.2 and 15 °C, these conditions resulted in the formation of MAA adducts enriched with the FAAB adduct (99%). Reaction of this BSA−FAAB adduct at pH 7.4 and 37 °C for 24 h with MDA generated substantial levels of BSA−MDHDC with a corresponding decrease in the levels of the FAAB adduct; however, when no MDA or when acetaldehyde was added under these conditions, minimal levels of the MDHDC adduct were formed (Table 1). Furthermore, when MDA was incubated with unmodified BSA, only slight MDHDC adduct formation was observed (Table 1). When the effects of concentration and time on the conversion of the FAAB adduct to the MDHDC adduct were examined, addition of MDA to the reaction mixtures at all concentrations tested resulted in substantial formation of the MDHDC adduct after 1 day of incubation, and formation of the MDHDC product continued up to 5 days of incubation, but at a decreased rate (Figure 2A). Also, MDA at concentrations of 5 and 10 mM produced similar yields of the MDHDC adduct which were higher than those observed for the 1 mM concentration (Figure 2A). On the other hand, when acetaldehyde was added to the reaction mixture containing the FAAB adduct, minimal formation of the MDHDC product was observed, and this was neither time nor concentration dependent (Figure 2A). Interestingly, decreases in the FAAB adduct were less pronounced in the presence of MDA compared to acetaldehyde or to the untreated sample (Figure 2B). A similar time- and concentration-dependent conversion of the FAAB adduct to the MDHDC adduct was also observed in the presence of MDA but not acetaldehyde when PLL−FAAB was treated with these aldehydes (Figure 3). Overall, these data indicate that the addition

Table 1. Effects of Incubation of Acetaldehyde or MDA with FAAB−BSA on the Formation of MDHDC−BSA

reaction conditions[a]	MDHDC[b]	FAAB[b]
FAAB-BSA (starting material)	1.7	136.4
FAAB-BSA + no additions (control)	6.5	31.1
FAAB-BSA + 2 mM acetaldehyde	6.2	36.4
FAAB-BSA + 10 mM acetaldehyde	5.1	30.9
FAAB-BSA + 2 mM MDA	21.2	48.2
FAAB-BSA + 10 mM MDA	33.9	50.4
unmodified BSA + 2 mM MDA	0.6	
unmodified BSA + 10 mM MDA	2.4	

[a] FAAB-BSA (10 mg/mL), prepared as described in the Experimental Procedures, or unmodified BSA (10 mg/mL) were incubated in phosphate buffer (pH 7.4) at 37 °C for 24h in the absence or presence of acetaldehyde or MDA. Following 24-h dialysis at 4 °C, the levels of FAAB-BSA and MDHDC-BSA were determined. [b] The data are expressed as nanomoles per milligram of BSA and represent the averages of two determinations.

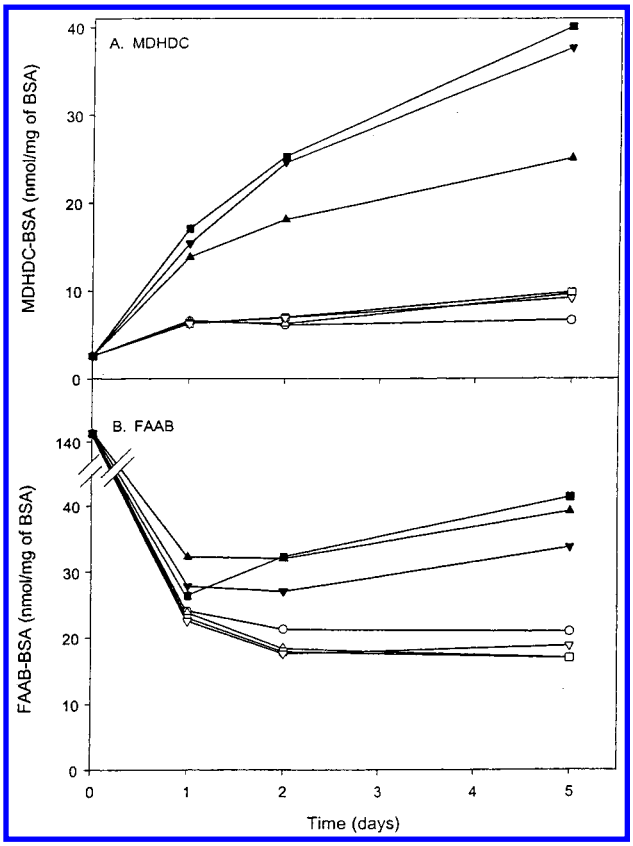

Figure 2. Effects of incubation of acetaldehyde or MDA with FAAB−BSA on the formation of MDHDC−BSA. FAAB−BSA (1 mg/mL), prepared as described in the Experimental Procedures, was incubated in phosphate buffer (pH 7.4) at 37 °C in the absence (○) or presence of 1 (△), 5 (□), and 10 mM (▽) acetaldehyde or 1 (▲), 5 (■), and 10 mM (▼) MDA for 1, 2, and 5 days. Following 24-h dialysis at 4 °C, the levels of MDHDC−BSA (A) and FAAB−BSA (B) were determined. The data are expressed as nanomoles per milligram of BSA and represent the averages of three determinations.

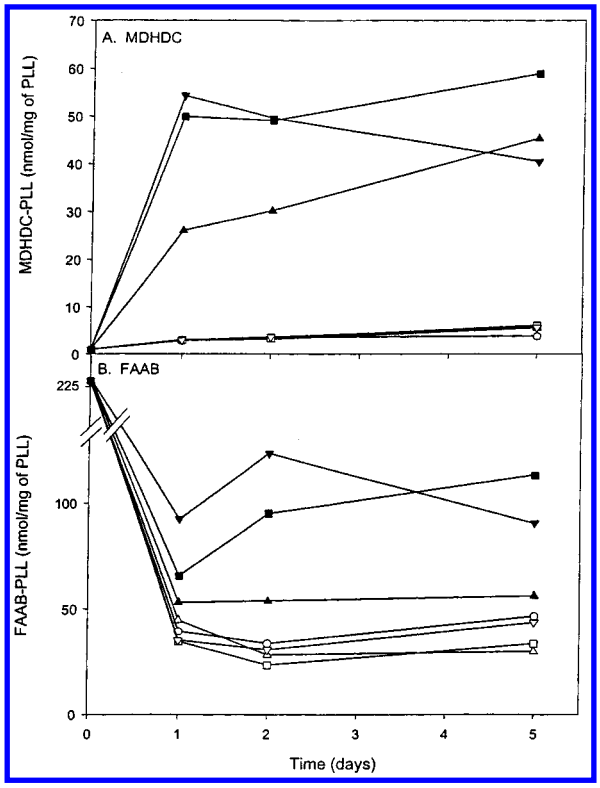

Figure 3. Effects of incubation of acetaldehyde or MDA with FAAB-PLL on the formation of MDHDC-PLL. FAAB-PLL (1 mg/mL), prepared as described in the Experimental Procedures, was incubated in phosphate buffer (pH 7.4) at 37 °C in the absence (○) or presence of 1 (△), 5 (□), and 10 mM (▽) acetaldehyde or 1 (▲), 5 (■), and 10 mM (▼) MDA for 1,2, and 5 days. Following 24-h dialysis at 4 °C, the levels of MDHDC-PLL (A) and FAAB-PLL (B) were determined. The data are expressed as nanomoles per milligram of PLL and represent the averages of three determinations.

of MDA to the preformed FAAB-adduct results in the conversion of this adduct to the MDHDC adduct.

Reaction of MAA Adducts (consisting of a mixture of FAAB and MDHDC adducts) with MDA. MAA adducts on BSA were generated so that 60% of the adducts were FAAB and 40% were MDHDC adducts. Unlike the results obtained with the BSA−FAAB enriched adduct, reaction of this mixed adduct with MDA did not result in increased formation of the MDHDC adduct. Instead, treatment with MDA or acetaldehyde or the untreated sample for 24 h of incubation resulted in small decreases (20−30%) in the levels of both the MDHDC and FAAB adducts (Table 2). Extending the times of reaction under these experimental conditions to 2 and 5 days did not appreciably change the results (data not shown). In addition, a mixed adduct of PLL (FAAB:MDHDC ratio of 60:40) behaved similarly to the mixed BSA adduct, yielding 20 to 30% decreases in both the MDA-, acetaldehyde-treated, and untreated reaction mixtures (data not shown).

Reactions of Hexyl-FAAB. The conversion of the FAAB adduct to the MDHDC adduct in the presence of MDA, which we observed for BSA− and PLL−FAAB, was studied in more detail using small/organic analogues of these adducts. By using an analogue such as hexyl-FAAB, we could conveniently monitor the conversion of this adduct to the MDHDC adduct by NMR spectroscopy. Hexyl-FAAB was dissolved into pD 7.4 buffer and its decomposition was followed for 24 h by ^{1}H NMR. For the FAAB adduct, the representative signals are due to

Table 2. Effects of Incubation of Acetaldehyde or MDA with MAA−BSA on the Formation of MDHDC−BSA

reactions conditions[a]	MDHDC[b]	FAAB[b]
MAA-BSA (starting material)	53.8	81.8
MAA-BSA + no additions (control)	42.1	60.0
MAA-BSA + 1 mM acetaldehyde	43.1	56.4
MAA-BSA + 5 mM acetaldehyde	42.8	57.7
MAA-BSA + 10 mM acetaldehyde	40.8	59.7
MAA-BSA + 1 mM MDA	40.6	58.9
MAA-BSA + 5 mM MDA	41.9	60.5
MAA-BSA + 10 mM MDA	40.2	62.4

[a] MAA-BSA (1 mg/mL), consisting of a mixture of FAAB-BSA and MDHDC-BSA, was prepared as described in the Experimental Procedures and was then incubated in phosphate buffer (pH 7.4) at 37 °C for 24 h in the absence or presence of various concentrations of acetaldehyde or MDA. Following 24-h dialysis at 4 °C, the levels of FAAB-BSA and MDHDC-BSA were determined. [b] The data are expressed as nanomoles per milligram of BSA and represent the averages of three determinations.

aldehyde, methine, and methyl protons which have chemical shifts of δ 8.6 (singlet), 4.2 (quartet), and 1.5 (doublet), respectively. The proton signals that indicate the presence of the MDHDC product are the aldehyde, vinyl, methine, and methyl protons which have chemical shifts of δ 9.2 (singlet), 7.3 (singlet), 3.7 (quartet), and 1.0 (doublet), respectively. After 24 h, hexyl-FAAB had decomposed significantly (Figure 4). The most significant new peak is at δ 3.0 (triplet) which is consistent with the formation of hexylamine. There were small signals at δ 9.2 (singlet) and 7.3 (singlet) which indicate that a small amount of hexyl-MDHDC was formed. In addition, there were many small signals between δ 8.0−9.0, which can be attributed to MDA-acetaldehyde products.[2]

Since the MDHDC product requires an extra mole of MDA, the hexyl-FAAB was allowed to react with MDA (Figure 5). For MDA, the representative ^{1}H NMR signal is due to the aldehydic protons with a chemical shift of δ 8.7 (singlet). Again, after 24 h hexyl-FAAB decomposed, but the MDHDC adduct was not a significant product. The major products were hexylamine, as evidenced by the signal at δ 3.0 (triplet), and 2,4-dihydroxymethylene-3-methylglutaraldehyde, a 2:1 MDA:acetaldehyde adduct shown in Figure 5. The representative signals for this glutaraldehyde are at δ 8.3 (singlet), 4.1 (quartet), and 1.2 (doublet) as reported by Gomez-Sanchez et al. (*23*). Just like the decomposition of hexyl-FAAB, there were small signals at δ 9.2 (singlet) and 7.3 (singlet) to show that a small amount of hexyl-MDHDC was formed.

Since MDA was ineffective in converting the FAAB adduct to the MDHDC adduct, we wondered whether MDA had to be in the form of a Schiff base in order to be reactive with the FAAB adduct; therefore, the reaction of hexyl-FAAB and *N*-ethylaminopropenal, the MDA−enamine of ethylamine, was monitored by ^{1}H NMR (Figure 6). After 24 h, more than half of the starting hexyl-FAAB had been converted to hexylamine and ethyl-MDHDC as evidenced by the new signals at δ 9.2 (singlet), 7.4 (singlet), and 1.1 (doublet). After 48 h, there was virtually complete conversion to MDHDC product. In the reaction of hexyl-FAAB and *N*-ethylaminopropenal, it is the nitrogen from the *N*-ethylaminopropenal that ends up in the MDHDC product. At δ 3.6−3.8, there are two quartets which correspond to the methine adjacent to the methyl and the methylene adjacent to the nitrogen. If the nitrogen from hexyl-FAAB had been in

[2] M.L.K., and D.J.T., unpublished observations.

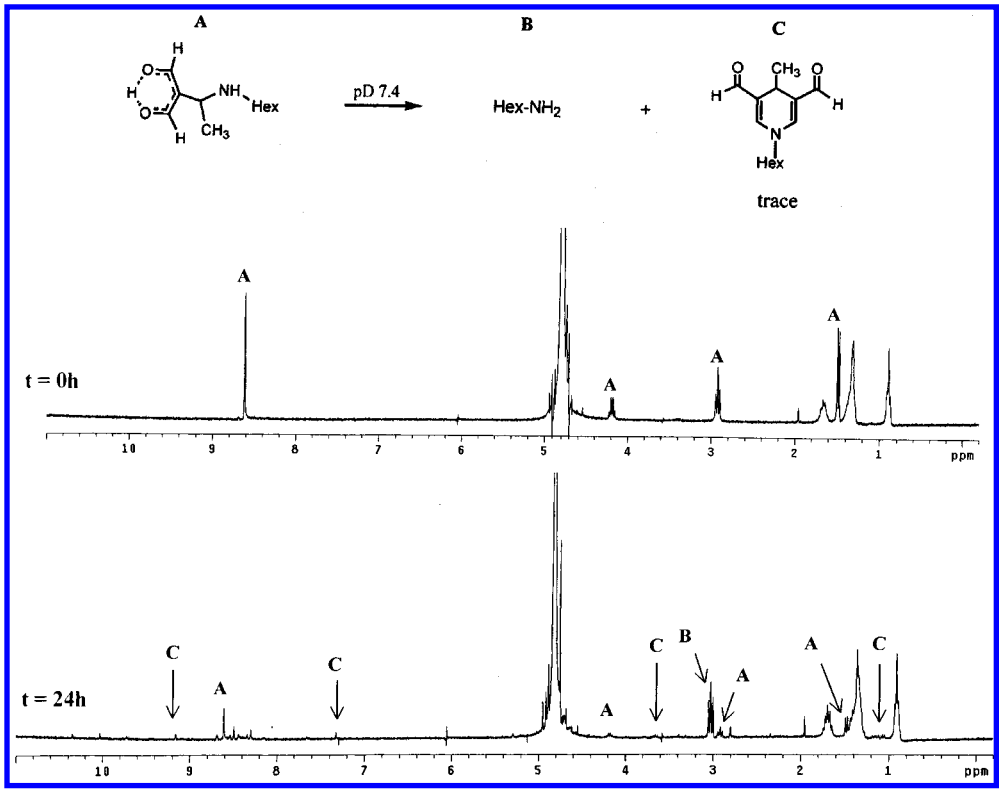

Figure 4. [1]H NMR spectra for the decomposition of hexyl-FAAB at pD 7.4. The signals correspond to the compounds as follows: hexyl-FAAB (A), hexylamine (B), and hexyl-MDHDC (C).

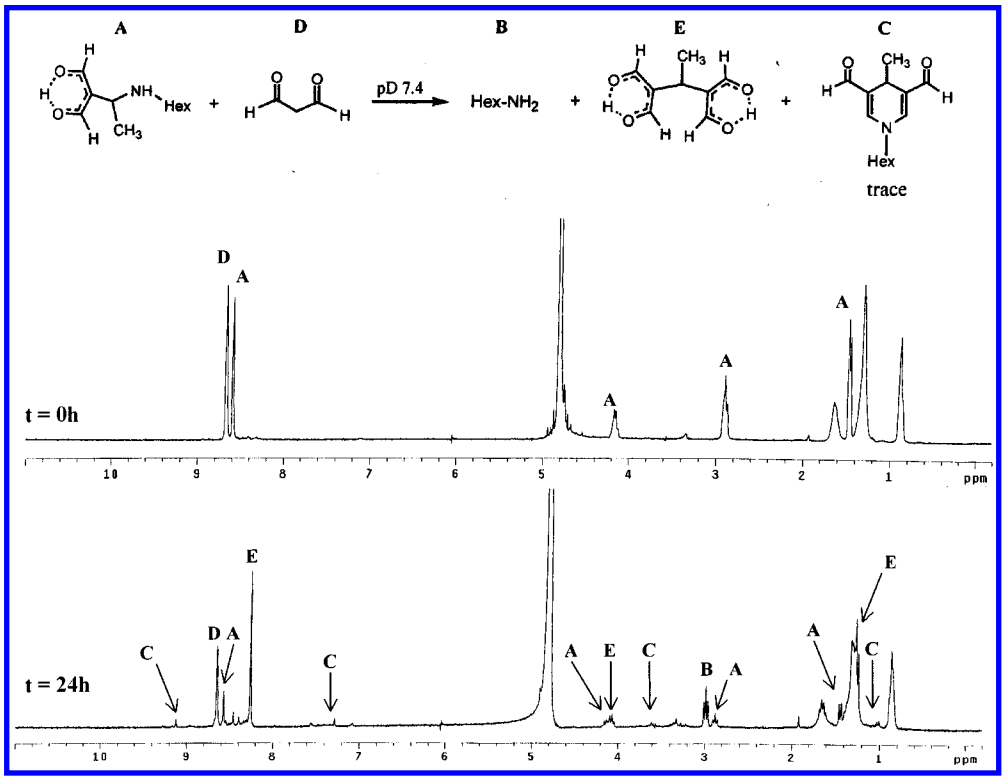

Figure 5. [1]H NMR spectra for the reaction of hexyl-FAAB and MDA at pD 7.4. The signals correspond to the compounds as follows: hexyl-FAAB (A), MDA (D), hexylamine (B), 2,4-dihydroxymethylene-3-methylglutaraldehyde (E), and hexyl-MDHDC (C).

the MDHDC, there would have been a quartet and a triplet at δ 3.6−3.8.

Overall, these data are in contrast to the data obtained for the polypeptide-FAAB adducts where addition of MDA readily converted this adduct to the MDHDC adduct. Instead, it takes the Schiff base derivative of MDA, but not MDA alone, to convert the smaller, organic derivative of FAAB (i.e., hexyl-FAAB) to the MDHDC product.

Reaction of FAAB−BSA or MAA−BSA with MDA or a Schiff Base-Derivative of MDA. Since the results obtained when hexyl-FAAB reacted with a MDA-Schiff base indicated that the FAAB moiety is transferred to the nitrogen of the MDA-Schiff base to form the MDHDC

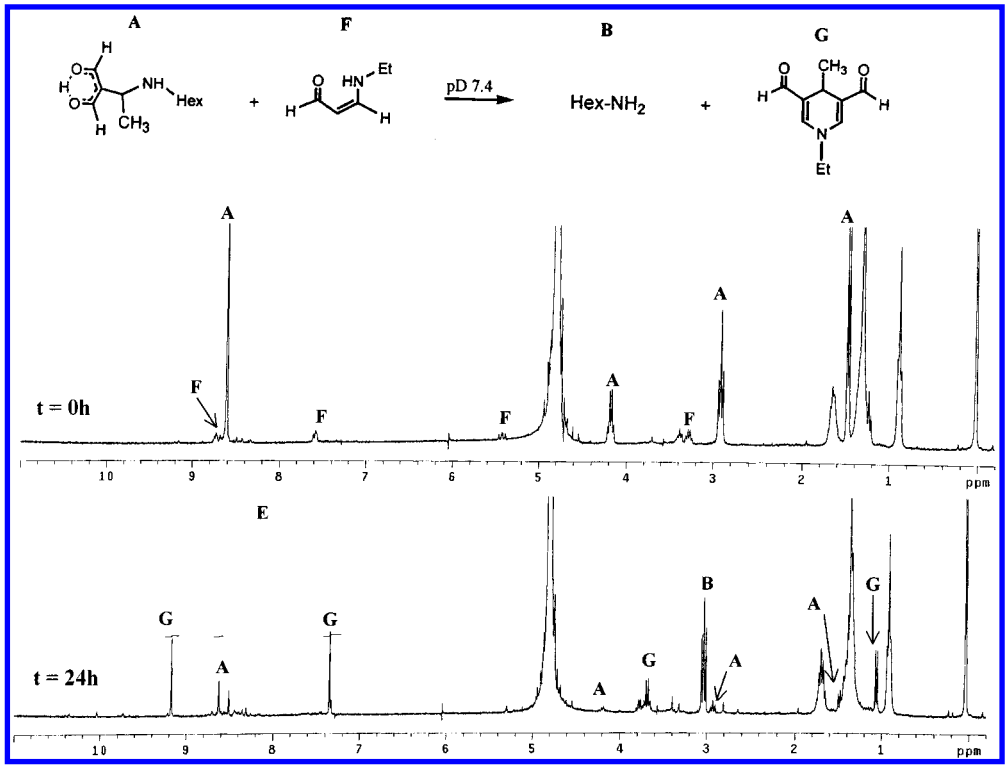

Figure 6. ^{1}H NMR spectra for the reaction of hexyl-FAAB and *N*-ethyl-aminopropenal at pD 7.4. The signals correspond to the compounds as follows: hexyl-FAAB (A), *N*-ethylaminopropenal (F), hexylamine (B), and ethyl-MDHDC (G).

adduct, the applicability of this mechanism to protein adducts was investigated. When FAAB−BSA was treated with MDA, only minor differences in the levels of the MDHDC adduct was observed before and after dialysis. However, treatment of FAAB−BSA with the MDA-Schiff base resulted in a marked reduction of the levels of the MDHDC adduct following dialysis (Figure 7A). Similarly, when the MAA−BSA (60% FAAB and 40% MDHDC) adduct was treated with MDA, dialysis did not alter the levels of the MDHDC product, but when treated with MDA-Schiff base we again observed a marked decrease in the MDHDC adduct in the retentate following dialysis (Figure 7B). Overall, these results indicate for MDA to convert the FAAB−BSA to the MDHDC adduct, the MDA must first form a Schiff base derivative with an amino group of the protein. Formation of this Schiff base allows for the transfer of the FAAB moiety to nitrogen of the Schiff base to form the MDHDC adduct which in this case is attached to the protein and hence resistant to dialysis. On the other hand, when a small, dialyzable Schiff base of MDA reacts with FAAB−BSA, the FAAB moiety is directly transferred to the small Schiff base, forming a small, dialyzable MDHDC product.

Reaction of Hexyl-FAAB with MDA−BSA Schiff Base. To further establish that the transfer of the FAAB moiety to the nitrogen of a MDA−Schiff base is an essential step in the formation of the MDHDC adduct, hexyl-FAAB was incubated with BSA which had been previously treated with MDA to generate Schiff bases on the protein. As shown in Table 3, treatment of this modified BSA with hexyl-FAAB resulted in significant formation of the MDHDC−BSA adduct, indicating transfer of the FAAB moiety from the small organic analogue to the protein. It should be noted that some MDHDC−BSA also formed when unmodified BSA was treated with hexyl-FAAB (Table 3).

Formation of MDHDC Adducts on Liver Proteins from Ethanol-Fed Rats Following Treatment with MDA. Our previous studies have shown the presence of MAA adducts on hepatic proteins from ethanol-fed rats (*5*). Later studies showed that the antibody used in the immunoassay to detect these MAA adducts specifically recognized only MDHDC epitopes (*11*); therefore, whether FAAB−protein adducts are present in the liver during ethanol consumption is still unknown. In view of these previous findings, the following experiments were designed to test the effect of in vitro treatment of liver homogenates obtained from ethanol-fed and control rats with MDA on the formation of MDHDC−protein adducts, and these results are shown in Figure 8. In confirmation of our previous study (*5*), elevated levels of MDHDC−protein adducts were detected in liver proteins from the ethanol-fed rats, and comparably very low levels (∼10% of ethanol-fed) were detected in the controls ($p < 0.01$). In vitro incubation of liver homogenates from the ethanol-fed animals with MDA increased MDHDC levels nearly 2-fold ($p < 0.05$) and also elevated the low levels of this adduct in the controls ($p < 0.02$). In addition, the specific inhibitor of antibody binding, hexyl-MDHDC, reduced the antibody response in all cases, indicating that the majority of the antibody response to liver proteins was attributable to the MDHDC adduct (Figure 8).

Since the FAAB adduct can dissociate during in vitro incubation, further experiments were conducted to minimize the effects of homogenization and subsequent sample handling on the breakdown of FAAB. Therefore, MDA was directly perfused into the livers prior to liver homogenization and sample processing. In this case, MDA treatment resulted in a marked increase in MDHDC adducts in livers from the ethanol-fed rats ($p < 0.01$) and also increased the low level of the adduct in the controls ($p < 0.01$)(Figure 9). Again, hexyl-MDHDC

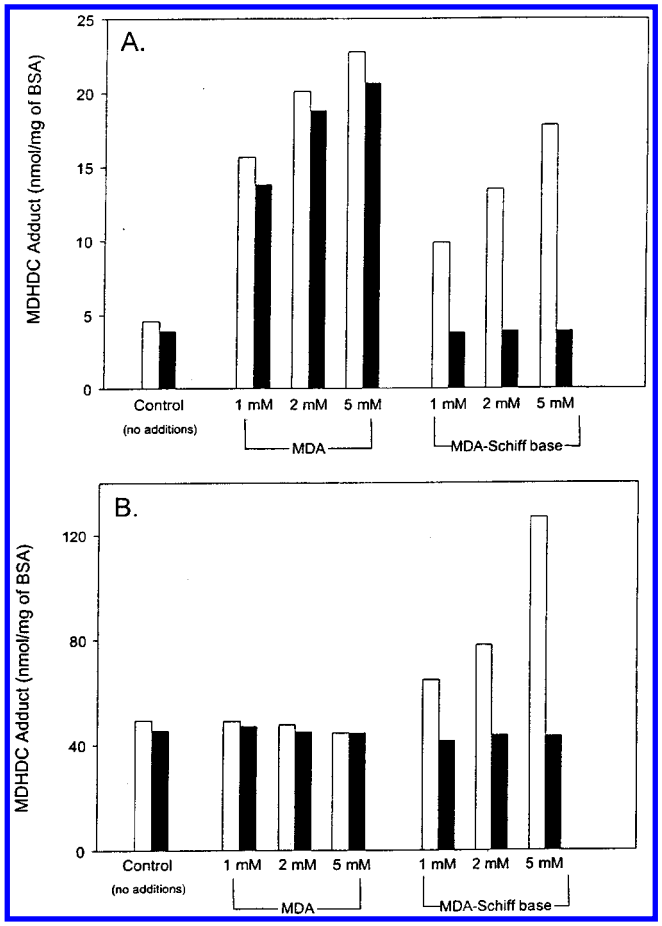

Figure 7. Reaction of FAAB−BSA or MAA−BSA with a soluble Schiff base derivative of MDA. (A) FAAB−BSA and (B) MAA−BSA (comprised of both FAAB and MDHDC adducts) at a concentration of 1 mg/mL BSA were allowed to react with various concentrations of MDA or a soluble Schiff base derivative of MDA (*N*-ethyl aminopropenal) at pH 7.4 and 37 °C for 24 h. Fluorescent measurements (indicator of MDHDC adducts) were conducted before (open bars) and after 24-h dialysis (closed bars). The data are expressed as nanomoles per milligram of BSA and represent the averages of two determinations.

Table 3. Effects of Incubation of Hexyl-FAAB with MDA-BSA Schiff Base on the Formation of MDHDC−BSA

	MDHDC-BSA[b]	
reactions conditions[a]	1-day incubation	2-day incubation
BSA-MDA Schiff base		
control (no additions)	0.93	1.33
+ MDA (1 mM)	1.33	1.70
+ MDA (5 mM)	2.19	3.12
+ hexyl-FAAB (1 mM)	26.30	50.60
+ hexyl-FAAB (5 mM)	59.20	76.40
unmodified BSA		
+ hexyl-FAAB (1 mM)	9.35	13.85

[a] MDA Schiff bases of BSA, prepared as described in the Experimental Procedures, or unmodified BSA at 1 mg/mL were incubated in phosphate buffer (pH 7.4) at 37 °C for 1 or 2 days in the presence of MDA or hexyl-FAAB. Following 24-h dialysis at 4 °C, the level of MDHDC−BSA was determined. [b] The data are expressed as nanomoles per milligram of BSA and represent the averages of two determinations.

effectively inhibited antibody binding (Figure 9). These data show that in vitro treatment of livers from ethanol-fed rats with MDA results in considerable formation of MDHDC−protein adducts, indicating the likely in vivo presence of FAAB adducts in livers of ethanol-fed animals.

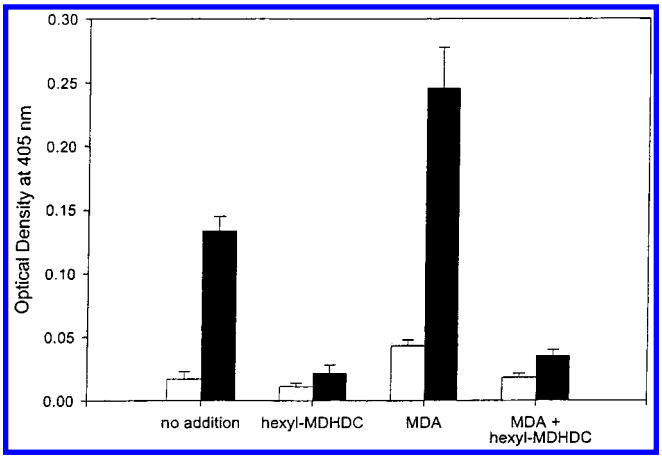

Figure 8. Effect of MDA on the formation of MDHDC epitopes on liver proteins obtained from ethanol-fed rats. Liver homogenates from pair-fed (open bars) and ethanol-fed (closed bars) rats were incubated in phosphate buffer (pH 7.4) at 37 °C for 24 h in the presence and absence of 1 mM MDA. An anti-MAA antibody, specific for the recognition of MDHDC epitopes, was used in an ELISA to detect the relative formation of MDHDC epitopes on liver proteins. ELISAs were conducted in the absence and presence of hexyl-MDHDC, a specific inhibitor of anti-MAA antibody binding. Values are expressed as means ± SE for four pairs of animals.

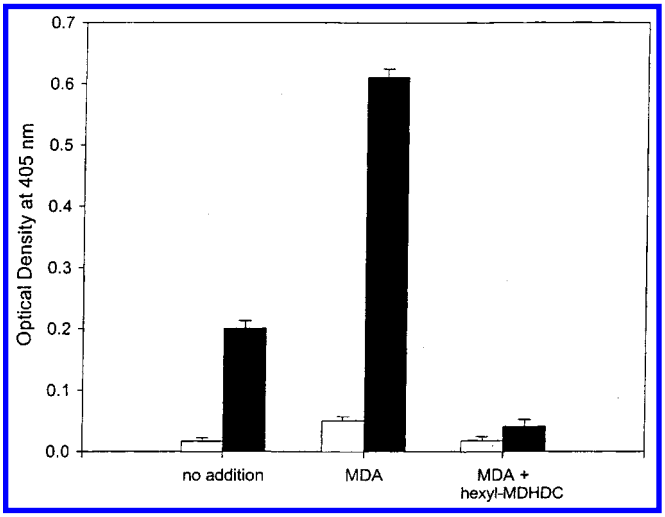

Figure 9. Effects of liver perfusion with a perfusate containing MDA on the formation of MDHDC epitopes on liver proteins. Prior to liver perfusion, two hepatic lobes were tied-off and removed, and ELISAs were conducted on homogenates from pair-fed (open bars) and ethanol-fed (closed bars) rats to obtain baseline values of MDHDC−protein epitopes (represented on figure as no additions). The remaining liver was perfused via the portal vein for 10 min with a perfusate containing 1 mM MDA. Liver homogenates were then prepared and incubated in phosphate buffer (pH 7.4) at 37 °C for 3 days in the presence of 1 mM MDA. ELISAs were performed to detect MDHDC epitopes on liver proteins as described in Figure 7. Values are expressed as means ± SE for 3 pairs of animals.

Discussion

MAA adducts, resulting from the reaction of proteins with acetaldehyde and MDA, have been detected in livers of ethanol-fed animals (*5*), and circulating antibodies against MAA adducts have been observed in ethanol-fed rats (*6*) and in patients with alcoholic liver disease (*7*). MAA adducts have been shown to be very immunogenic (*8*) and to possess pro-inflammatory and profibrogenic properties (*9*, *10*). Our previous studies demonstrated that MAA adducts are comprised of two major products (*11*, *12*), the FAAB adduct and the MDHDC adduct

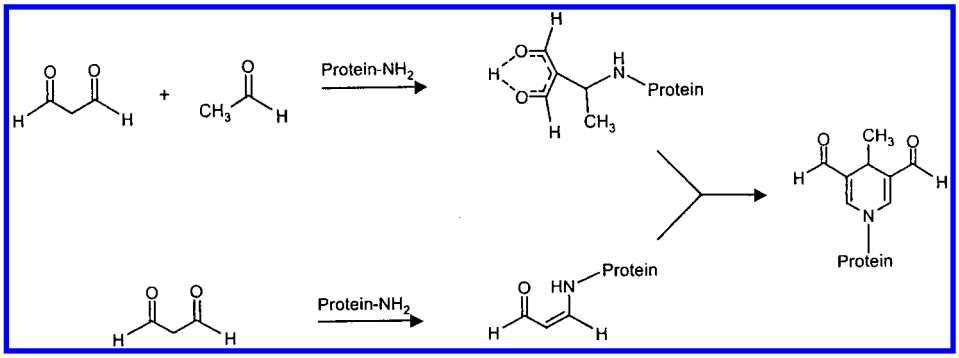

Figure 10. Proposed reaction scheme for the formation of MDHDC–protein adducts.

(Figure 1). It should also be pointed out that in addition to proteins MDHDC and FAAB-like adducts have also been shown to form with DNA (*24*, *25*). The purpose of the present study was to clarify the chemistry of MAA adduct formation, especially focusing on whether the FAAB adduct serves as a precursor for the generation of the MDHDC adduct, and to gain information concerning the relevance of this reaction in vivo.

Based on the present findings, a reaction scheme describing the formation of the MDHDC–protein adduct is proposed and illustrated in Figure 10. Two initial reaction steps appear to be a prerequisite to MDHDC generation. One step involves the reaction of one molecule of MDA and one of acetaldehyde with an amino group of a protein to form the FAAB adduct. The other step involves the reaction of MDA with another amino group to form a MDA Schiff base (MDA–enamine). Reaction of these two intermediates results in the formation of the MDHDC adduct.

Several lines of experimental evidence support this proposed reaction scheme. Previous studies showed that, during the reaction of proteins with MDA and acetaldehyde, FAAB adduct formation precedes the formation of the MDHDC adduct (*11*, *12*), an observation consistent with a precursor-product relationship. Furthermore, MDA-Schiff base formation is a prominent product when MDA reacts with proteins (*17*, *18*). Combined together, these studies clearly establish the presence of these two proposed intermediates prior to the generation of the MDHDC adduct.

The results of the present study support the occurrence of the key step of the reaction scheme, which is the apparent "transfer" of the FAAB moiety to the nitrogen of the MDA–enamine. First of all, addition of MDA to a preformed FAAB–protein adduct resulted in a significant conversion of this adduct to the MDHDC–protein adduct. However, in the case of the small, organic FAAB analogue (i.e., hexyl-FAAB), the addition of the MDA–enamine (but not free MDA) was necessary to form the MDHDC adduct. The most likely explanation of these differences in reactivity with free MDA is that in the case of hexyl-FAAB, there is a lack of availability of free amino groups to form MDA–enamines; whereas, with proteins an ample number of amino groups are available for MDA–enamine formation.

When considering the mechanism of formation of the MDHDC–protein adduct, it is necessary to refer to studies of the classical Hantzsch reaction because the MDHDC adduct is an example of an Hantzsch adduct. The Hantzsch reaction has been studied by numerous groups and there are a number of plausible mechanisms that have been proposed (*23*, *26*, *27*). All of the proposed mechanisms require 2 mol of MDA and 1 mol each of acetaldehyde and a primary amine, but they differ with respect to the intermediate species. However, none of the proposed mechanisms invoke the FAAB adduct as an intermediate. In fact, it is surprising, at first consideration, that the FAAB adduct would serve as a precursor to the MDHDC adduct because in FAAB the acetaldehyde and amine are connected; whereas, in MDHDC the acetaldehyde is attached to both molecules of MDA. Thus, either the FAAB adduct dissociates prior to the formation of MDHDC or the FAAB and MDA–enamine react in an unusual concerted reaction.

If the dissociation pathway is at play, there are two probable mechanisms as shown in Figure 11. FAAB most likely initially dissociates into free amine and intermediate I (although this reactive intermediate was not observed in the NMR spectra), and this intermediate can follow two paths of reaction to generate the MDHDC adduct (Figure 11). Studies by Nair et al. (*26*) support path A as a possible mechanism for MDHDC formation, whereas several groups have indicated that path B may be the major mechanism (*23*, *26*, *27*). Of the two mechanisms shown, our evidence supports path A. In the hexyl-FAAB decomposition experiments (Figures 4 and 5), at 24 h there was very little MDHDC produced in the absence or presence of MDA. However, when hexyl-FAAB was allowed to react with MDA–enamine, MDHDC was a significant product at 24 h (Figure 6). Thus the second mole of MDA was from the MDA–enamine (path A) and not from free MDA (path B). Further experiments with proteins also support path A and the participation of the MDA–enamine. When a small, organic Schiff base of MDA (*N*-ethylaminopropenal) was added to BSA–FAAB, MDHDC adduct did not form on the protein but instead a small, dialyzable MDHDC adduct was generated. Presumably, this MDHDC was formed on the nitrogen of the MDA–enamine. Conversely, when BSA was allowed to form Schiff bases with MDA, treatment of this derivatized BSA with hexyl-FAAB resulted in the MDHDC adduct being present on the protein. Overall, these data indicate the necessity of both FAAB and MDA–enamine for the formation of the MDHDC adduct on BSA. Further studies are currently in progress to further elucidate the mechanistic details of the transfer of the FAAB moiety to the nitrogen of the MDA–enamine.

As expected, the levels of the FAAB adduct decreased when preformed BSA–FAAB (Figure 2B) and PLL–FAAB (Figure 3B) were treated with MDA during formation of the MDHDC adduct. However, decreases in FAAB exceeded the formation of the MDHDC product, indicating that the transfer of the FAAB moiety to the MDA–enamine of proteins was not quantitative. Although the

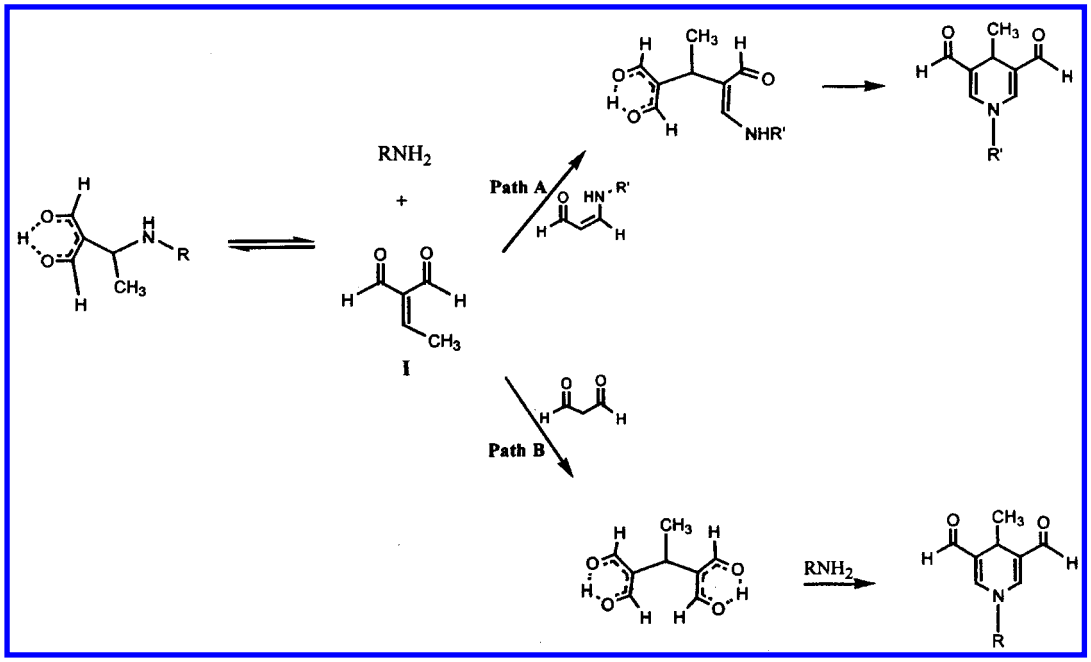

Figure 11. Reaction schemes describing the conversion of the FAAB adduct to the MDHDC adduct.

conversion of the FAAB adduct by treatment with a MDA−enamine to the MDHDC product essentially went to completion when small organic analogues were used, the transfer of FAAB to the MDA−enamine appears to be much less efficient for proteins. Apparently with proteins some of the FAAB dissociates and perhaps decomposes before it can react with the MDA−enamine. This dissociation, coupled with the observation that FAAB can decompose and reform the aldehydes, could also explain why low levels of MDHDC adducts are formed when preformed FAAB adducts are incubated alone at 37 °C or when incubated with acetaldehyde. Related to this is the observation that decreases in FAAB adduct were less pronounced in the presence of MDA compared to the acetaldehyde-treated or untreated samples. Either MDA can stabilize the FAAB adduct or, more likely, some FAAB adduct can reform after dissociation in the presence of excess MDA.

An interesting and important feature concerning the transfer of the FAAB moiety to the MDA−enamine was noted when the extent of this transfer reaction was compared in proteins with different ratios of MAA adducts. In the case of MAA adducts enriched in the FAAB product (99%), addition of MDA to this adduct caused a significant formation of the MDHDC adduct; whereas, MAA adducts, consisting of a 60 and 40% mixture of FAAB and MDHDC adducts, respectively, did not generate more MDHDC adducts upon addition of MDA. Apparently, when preformed MDHDC adducts are present, available reaction sites are already occupied by the MDHDC; therefore, these sites are unavailable to form the MDA−enamine and accept transfer of the FAAB moiety. If the number of amino groups, occupied by either the FAAB or MDHDC adduct in the starting material (MAA adducts composed of a 60:40 ratio of FAAB to MDHDC), is calculated, about nine amino groups are occupied by these adducts, leaving about 50 amino groups still available to potentially react. Therefore, the failure of the FAAB transfer reaction in this case cannot be merely due to the lack of available free amino groups. Instead, it appears that there is some selectivity in this transfer reaction and that the position of the FAAB adduct relative to the MDA−enamine in the protein is an important determinant for the efficient transfer of the FAAB moiety to the MDA−enamine and subsequent formation of the MDHDC product. This would imply that an important factor in governing the formation of the MDHDC adduct in a given protein would be the ability of the protein to not only form the necessary intermediates (i.e., FAAB and MDA−enamine), but to form them in the appropriate relative positions of close proximity to each other to allow for the efficient transfer of FAAB to the MDA−enamine. This consideration would explain our previous findings which showed a wide variation in MDHDC adduct formation among various proteins tested and further showed that the extent of MDHDC adduct formation for a protein was not dependent simply on the number of amino groups (*11*). Therefore, proteins which are capable of generating the FAAB and MDA−enamine intermediates in the proper location on the peptide chain would be selective targets of MDHDC adduct formation. This factor could potentially be helpful in identifying protein targets for MDHDC adduct formation in vivo during ethanol consumption.

In the final set of experiments, information was obtained concerning MAA adduct formation in the in vivo situation during ethanol consumption in the rat. Our previous studies demonstrated the presence of MDHDC−protein adducts in livers from ethanol-fed rats (*5*, *11*); however, to date, we have been unable to obtain an appropriate antibody that recognizes the FAAB adduct. Therefore, the formation of the FAAB adduct in the liver during ethanol consumption could not be ascertained. Our current results showed that in vitro incubation of liver homogenates from ethanol-fed rats with MDA markedly increased the levels of MDHDC−protein adducts, indicating indirectly that FAAB adducts are likely present in livers from ethanol-fed animals. Furthermore, these data suggest that MDHDC−protein adduct formation in vivo may very well proceed by the same reaction scheme as shown in Figure 10. Interestingly, in the pair-fed controls, a low signal indicating the presence of

MDHDC adducts was observed. The ability of MDA treatment to further elevate the levels of MDHDC adducts was also observed, indicating the presence of FAAB adducts in control livers as well. These data suggest that both types of MAA adducts are present in the livers of controls, but at very low levels compared to the ethanol-fed animals.

In conclusion, our present studies show that MDHDC−protein adduct formation occurs via the reaction scheme illustrated in Figure 10 and further suggest that MDHDC adduct formation in ethanol-fed rats may also proceed by such a reaction pathway. Extrapolation of these findings, that are relevant to the role of MAA adducts in the pathogenesis of alcoholic liver injury, allows us to propose a series of events that could take place during ethanol consumption that would lead to liver injury. In the early stages of ethanol consumption, FAAB adduct formation would be favored because acetaldehyde levels would likely exceed those of MDA. This situation would result in a pool of FAAB adducts in the liver. With more prolonged ethanol intake, which has been shown to induce oxidative stress (*3*, *4*, *28*, *29*) and elevate substantially the levels of MDA (*30*, *31*), increased MDA would be available to generate Schiff base adducts with proteins, and subsequently these Schiff bases would be available to accept the transfer of the FAAB moiety and form MDHDC adducts. Elevated levels of MDHDC−protein adducts, which have been shown to be very immunogenic (*8*) and to possess pro-inflammatory[3] and profibrogenic[3] properties (*9*, *10*), could by virtue of these potential toxic effects contribute to the pathogenesis of alcoholic liver injury.

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