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A Structural Assignment for a Stable Acetaldehyde-Lysine Adduct*

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Acetaldehyde is the first oxidation product of ethanol *in vivo*. Lysine residues in proteins such as hemoglobin have been implicated as target structures for acetaldehyde adducts resulting from ethanol consumption. Although the presence of both stable and unstable acetaldehyde-hemoglobin adducts has been established, the structural characterization of the adducts has received relatively little attention. As a model for such adduct formation, we studied the peptide pentalysine *in vitro*. Pentalysine has several potential sites for adduct formation. The amino-terminal amine group as well as the ε-amine groups of each lysine side chain can serve as potential sites for modification by acetaldehyde. Mass spectrometry, nuclear magnetic resonance, and Raman spectroscopy were employed to demonstrate that acetaldehyde forms a stable linkage to lysine amine groups via a Schiff base.

The development of more effective forms of treatment for alcohol use and abuse has been hampered by the lack of a firm biochemical basis with which to explain the observed physiological conditions pertaining to alcoholism. A large step toward understanding the relevant biochemistry occurred with the discovery that hemoglobin and other proteins react with acetaldehyde, the major oxidation product of ethanol *in vivo*, to form a number of stable and labile protein-acetaldehyde adducts (1, 2). These adducts not only have been implicated in some of the resulting complications of alcoholism but also may serve as useful markers of ethanol intake (3–7).

The posttranslational modification of proteins has been recognized as playing a role in the subsequent pathology of many diseases. For example, cyanate, from the breakdown of urea in persons in renal failure, can lead to neuropathy associated with demyelination and protein carbamylation (8). Complications of diabetes mellitus have been attributed to nonenzymatic glycosylation of proteins (9). Glycation of hemoglobin in diabetes has been shown extensively and provides a useful clinical marker of glucose control (10). Maillard products also have been implicated in the aging process (9).

Recent experiments indicate that acetaldehyde forms stable adducts with several biomolecules including hemoglobin (11), plasma proteins and albumin (12), P450 (13), nucleosides (14), plasma membrane proteins (15), microtubular proteins and tubulin (16, 17), low density lipoproteins (18), and ribonuclease (19). The clinical use of these potential markers of ethanol exposure and their pathological implications would be aided by a complete structural characterization of the putative protein-acetaldehyde adducts.

Tuma *et al.* (20) used [^{14}C]acetaldehyde in reactions with proteins such as albumin and polylysine. The radiolabeled hydrolysis products contained similar ratios of stable to unstable adducts for both albumin and polylysine, suggesting that lysine was the primary residue involved in acetaldehyde binding *in vivo*. Ding and co-workers (21) showed that lysine residues of serum albumin were the positions where modification by tolmetin glucuronide formed stable adducts via an imine intermediate. Also using ^{14}C-labeled acetaldehyde adducts of hemoglobin, San George and Hoberman (22) concluded that a tetrahedral imidazolidinone derivative was formed for non-borohydride-reducible adducts. Additional experimental evidence suggests, however, that several different adducts may be formed in addition to the imidazolidinone structure (23). A complete structural analysis of acetaldehyde adducts similar to those thought to form *in vivo* has not been undertaken. We therefore initiated structural studies with a model compound, pentalysine.

MATERIALS AND METHODS

Chemicals—Pentalysine was purchased from Bachem Bioscience. Cyclohexanedione (CHD)[1] was purchased from Aldrich. [1,2-^{13}C]Acetaldehyde was obtained from Cambridge Isotope Laboratories (Andover, MA). NMR solvents were obtained from Aldrich. Materials were used as received from the manufacturer with no additional purification except where noted.

Purification of Pentalysine—The model peptide pentalysine was purified first by passing the compound through a C_{18} Bond-Elut Pak with 10% acetonitrile, followed by reversed-phase high performance liquid chromatography (HPLC). The solvent system consisted of acetonitrile and water in a linear gradient from 10 to 60% acetonitrile over 15 min. Acetic acid (0.1%) was added to aid peak resolution.

Determination of Acetaldehyde Concentration—Acetaldehyde concentration was determined using the method of Peterson and Polizzi (24). The assay is based on the procedure of Stahovec and Mopper (25) for quantification of aldehydes. A 1-ml solution of peptide (1 mM) was incubated with 1.5, 5, or 8 mM acetaldehyde for 1 h at 37 °C. Residual free acetaldehyde was removed by ultrafiltration in 3 volumes of 1.5 ml of H_2O and a YC05 500 M_r cutoff membrane (Amicon, Beverly, MA) prior to analysis.

CHD solution was prepared by dissolving 400 mg of 1,3-cyclohexanedione, 10 g of NH_4COOCH_3, and 3.2 ml of concentrated HCl in 30 ml of H_2O. Contaminants were removed by heating the solution in a sealed bottle at 70 °C for 1 h and passing the cooled solution through a C_{18} Bond-Elut cartridge (Analytichem, Harbor City, CA).

The CHD reagent was calibrated with acetaldehyde by the measurement of prepared acetaldehyde standards as follows. 1 ml of cleaned reagent was added to 1 ml of varying concentrations of acetaldehyde (0, 0.25, 0.5, 1.0, 2.5, 5.0, 8, or 16 μM) in a 1.5-ml screw cap centrifuge tube (Sarstedt) and allowed to react for 1 h at 70 °C. The reaction was stopped by placing the sample in an ice bath until analyzed by HPLC.

Chromatography was performed on a Gilson system equipped with a 250 × 4.6-mm C-18 column (ultrasphere ODS) and pellicular guard. Chromatograms were obtained by isocratic elution with H_2O/acetoni-

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[1] The abbreviations used are: CHD, cyclohexanedione; HPLC, high performance liquid chromatography; MS, mass spectrometry.

trile (70:30) (v/v) and a flow rate of 1 ml/min. Peaks were detected by measurement of fluorescence with a 305–395-nm excitation filter and a 450 ± 3.5-nm emission filter. Peak integration was accomplished with a Gilson system 715 peak integrator. Results were read as micromolar acetaldehyde as determined from the standard curve of acetaldehyde concentrations.

Mass Spectrometry—Electrospray ionization spectra were obtained using a mass spectrometer (SX505, JEOL, Peabody, MA) equipped with liquid chromatography/mass spectrometry (MS) electrospray apparatus. A minimum mass of 2000 atomic mass units was used. Each sample (see above) was diluted 1:1 with acetonitrile/water/acetic acid (75:25:1). For some spectra, direct injection onto the mass spectrometer was used, bypassing the liquid chromatography apparatus. A 2-μl injection of sample was eluted into a 600 μl/min stream of acetonitrile/water/acetic acid (75:25:1) split 150:1 into the electrospray interface. Drying gas was maintained at 200 °C. Data were acquired over a range of 100–600 atomic mass units with a scan threshold of 50 atomic mass units.

Fast atom bombardment spectra were obtained on a model HX110 mass spectrometer with forward geometry and a double focusing 6-kV xenon primary beam and an accelerating voltage of 10 kV. Scans from 0 to 2200 atomic mass units were made in 18 s. Additional electrospray ionization spectra were obtained on a model SX102 mass spectrometer with a source-accelerating voltage of 5 kV, a reverse geometry double focusing mass spectrometer, and a flow rate of 2 μl/min. The sample was diluted 1:1 with H_2O/methanol and 2% acetic acid. The ion source was operated at room temperature. Nitrogen drying gas was heated to 180 °C and introduced at a flow rate of 5 liters/min.

Tandem MS/MS spectra were obtained by infusing the sample in electrospray ionization solvent (acetonitrile/water/acetic acid (75:25:1)) at 1 μl/min. Doubly charged precursor ions were selected and attenuated to approximately one-third of the original abundance by the addition of helium to the collision cell. Linked scans were accomplished by dividing the accelerating voltage by 2 so that the doubly charged ions had a total of 10 kV of kinetic energy.

NMR Spectroscopy—Proton-decoupled ^{13}C nuclear magnetic resonance spectroscopy was performed on a Bruker AC-300 spectrometer operating at a spectral frequency of 75.47 MHz and a temperature of 25 °C. A 45° pulse and 5-s delay between acquisitions were used at a resolution of 1.1 Hz/point. Water with 5% D_2O was used as solvent. A total of 6500 scans was acquired for each peptide sample, and 98 scans were acquired for the acetaldehyde sample. Chemical shifts were reported based on an internal reference of *N,N*-dimethyl sulfoxide.

Raman Spectroscopy—Raman spectra were acquired using a Spex 1401 double monochromator (Metuchen) equipped with 1000-μm slits, a coherent argon ion laser tuned to 514.5 nm, and an average of 80 scans/sample. All spectra were obtained on neat sample or aqueous solution in glass capillary tubes. Both acetaldehyde-treated and nontreated peptides were analyzed to provide a basis for comparison.

RESULTS

Acetaldehyde Concentration—Acetaldehyde from pentalysine adducts stable to ultrafiltration conditions (see "Materials and Methods") was quantified by the fluorimetric assay. The sample prepared with 0 mM acetaldehyde contained no measurable CHD-reactive acetaldehyde, the sample prepared with 1.5 mM acetaldehyde was determined to contain 0.95 mM CHD-reactive acetaldehyde, and the 5 and 8 mM samples were both determined to contain 3.3 mM CHD-reactive acetaldehyde. Incubation acetaldehyde concentration *versus* concentration of recovered acetaldehyde after adduct formation and ultrafiltration is demonstrated in Fig. 1. The relationship between added acetaldehyde and recovered acetaldehyde is linear for all acetaldehyde concentrations except the highest value (8 mM), indicating that no additional sites on the pentalysine molecule are modified with the higher concentrations of acetaldehyde.

Mass Spectrometry—When a 1.5:1 mole ratio of acetaldehyde to peptide was used, a monoadduct is observed (data not shown). Likewise, the mass spectrum of the model peptide pentalysine following incubation with 20 μM acetaldehyde showed a single acetaldehyde adduct at a mass of 684 atomic mass units. However, when an excess of acetaldehyde was used, the number of adducts observed increased. For the 8:1 mole ratio acetaldehyde/peptide mixture, five different adducts are observed with masses of 684, 710, 736, 762, and 788 atomic mass units. The difference in mass in each case indicates a structure consistent with a Schiff base (a difference of 26 atomic mass units as shown in Fig. 2, peaks *A–E*). Of particular interest is the decreasing relative abundance of adduct with the increasing number of Schiff base adducts/molecule. In the sample shown, 40% of the peptide sample molecules have at least 1 acetaldehyde adduct, approximately 25% of the sample molecules have at least 2 adducts, 20% of the molecules have at least 3 adducts, and less than 10% of the sample molecules have 4 or 5 adducts.

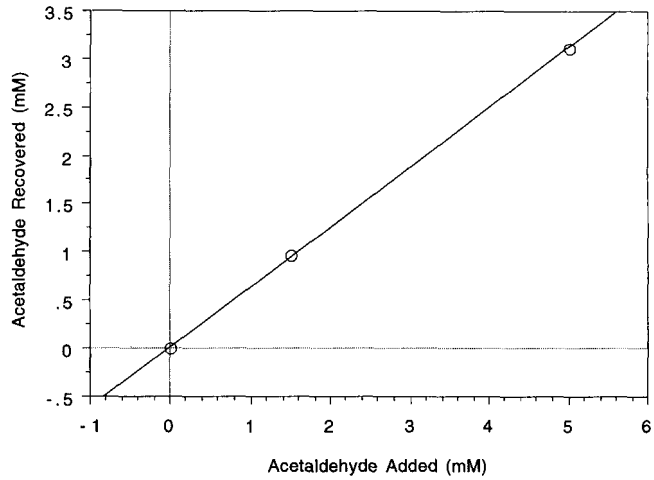

FIG. 1. **Incubation concentration of acetaldehyde compared with recovered acetaldehyde after incubation with pentalysine.** Recovery of acetaldehyde from pentalysine after incubation and ultrafiltration is 66% of the added amount in each sample except the 8 mM sample, where recovery is 41% of the added amount. The equation for the regression line, excluding the 8 mM sample, is $y = 54.8 + (5901.6)x$ ($R^2 = 1.00$).

Tandem MS/MS spectra were obtained to ascertain the point(s) of acetaldehyde adduct formation (Table I). For these experiments, pentalysine-acetaldehyde adducts were generated by the addition of acetaldehyde in an 8:1 mole ratio (acetaldehyde to peptide). The pattern of y- and b-type fragment ions (y_4 + 26 and b_1 + 26 atomic mass units) is strongly suggestive of modification at the first and second positions at the amino terminus of the peptide. Weak b and y fragments for residues 3, 4, and 5 suggest but do not confirm the presence of modifications on those side chains.

NMR Spectroscopy—In order to confirm the Schiff base structure, [1,2-^{13}C]acetaldehyde was used to generate adducts with pentalysine. NMR spectra of the peptide-acetaldehyde adduct show the presence of signals at 155–156 ppm consistent with Schiff base structures between the lysine side chain amino groups and the ^{13}C-labeled acetaldehyde. Comparison of the spectra of acetaldehyde alone to the peptide-acetaldehyde adduct shows that the aldehyde resonance at 202 ppm disappears, and the resonances at 155–156 ppm appear in the adduct sample (see Fig. 3), consistent with a Schiff base product.

Raman Spectroscopy—The Raman spectra were obtained to provide further proof of the imine structure (Fig. 4). The spectrum shows a strong peak at approximately 1640 cm^{-1}. This peak is consistent with literature values for imine C=N structures (26). The peak at approximately 1450 cm^{-1} is presumably due to C–H deformation modes that originate from all amino acid residues (27).

DISCUSSION

Our study confirms a Schiff base adduct of pentalysine and acetaldehyde that is stable to our preparative and analytical procedures. The identification of stable Schiff bases in bioactive

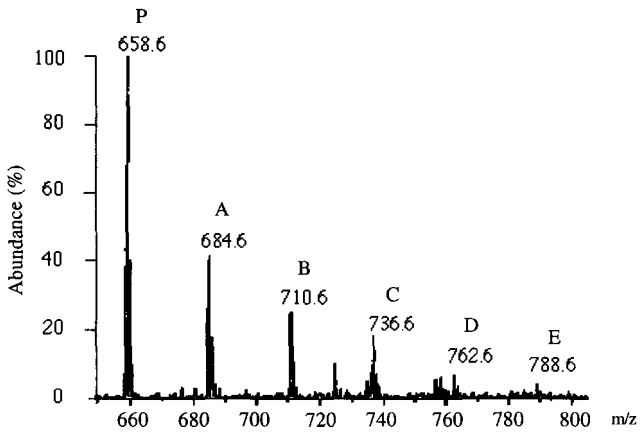

FIG. 2. **Mass spectrum of multiple adducts of pentalysine and acetaldehyde.** Sample was prepared with an 8:1 mole ratio of acetaldehyde to pentalysine. The *letters* indicate the following: *P*, pentalysine; *A–E*, adducts of acetaldehyde-peptide.

TABLE I
Observed ion fragments from MS/MS analysis based on theoretical calculations of expected fragments

Residue	b ion	b ion adduct	y″ ion	y″ ion adduct
1	129.10	155.08	147.11	
2	257.20	282.97	275.21	
3	385.29		403.30	429.41
4	513.39		531.40	557.37
5	641.48		659.49	

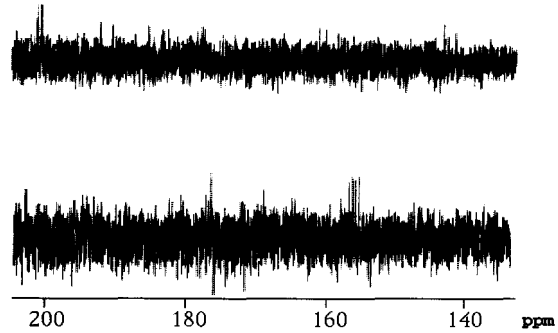

FIG. 3. **^{13}C nuclear magnetic resonance spectra of 1,2-[^{13}C]acetaldehyde in deuterochloroform (*top*) and 1,2-[^{13}C]acetaldehyde bound to pentalysine (*bottom*).** Sample was prepared with an 8:1 mole ratio of acetaldehyde to peptide.

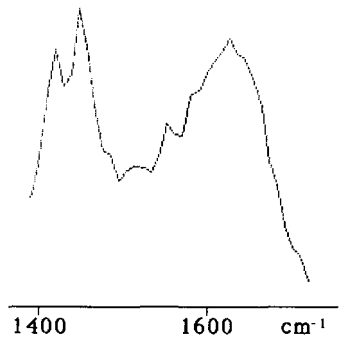

FIG. 4. **Raman spectrum of acetaldehyde-pentalysine adducts, documenting imine peak at 1640 cm^{-1}.** Sample was prepared with an 8:1 mole ratio of acetaldehyde to peptide.

molecules is currently of great interest, and the role of the imine is often critical to bioactivity (21, 22, 28, 29). For example, enzyme-substrate intermediates such as that formed between porphobilinogen synthase and 5-aminolevulinic acid incorporate a Schiff base linkage that is stable to analysis (30).

Although much interest in the structural features of acetaldehyde-blood protein adducts has been expressed in the literature, attention has generally focused on borohydride-reduced adducts. This approach is due in part to the complicated chemistry of acetaldehyde adduct formation. Several possibilities for subsequent reaction after initial adduct formation exist (31). For example, after Schiff base formation, subsequent reaction with another reactive group such as a thiol can result in addition across the double bond to form a stable product. Either inter- or intramolecular cross-linking of protein chains via acetaldehyde adducts can occur, although this would occur through a different mechanism from the Amadori rearrangement of glycated proteins (31). Other reaction schemes are possible; suggestions have been made that at least two different types of acetaldehyde adducts are formed at the amino termini of proteins and digest peptides (23). Our study supports the formation of stable Schiff bases with lysine residues at physiological as well as superphysiological concentrations of acetaldehyde.

Additional studies of the model peptide incorporated acetaldehyde in a physiologically relevant (20 μM) concentration. Mass spectra obtained with electrospray ionization and fast atom bombardment techniques showed a single acetaldehyde-peptide adduct (mass, 684 atomic mass units; native peptide, 658 atomic mass units) with relative peak heights in a ratio of 11:1 for the native peptide *versus* adduct (data not shown). The spectrum of the 1.5:1 mole ratio acetaldehyde-peptide showed a monoadduct (mass, 684 atomic mass units) as well, with relative peak heights of native peptide to adduct of 1:1.3. The mass difference between native and adduct molecules in both cases is consistent with a Schiff base between an amine and acetaldehyde. None of the data are consistent with cross-linking of the peptide adducts even at the highest concentrations of acetaldehyde used to generate adducts. Mass spectra show no masses consistent with fragments from either intra- or interpeptide cross-linking. In addition, the modified peptide chromatographs as a single peak on a C-18 reversed phase column, where cross-linking of some of the molecules would change chromatographic behavior sufficiently to generate an additional peak (data not shown).

The identification of these Schiff base adducts has several implications for the study of ethanol oxidation products *in vivo*. We have demonstrated that stable Schiff bases can occur between acetaldehyde and amino acids such as lysine. The spectroscopic data presented here suggest that these Schiff bases are stable, preferred structures for adduct formation in systems such as our model peptide. We cannot eliminate the possibility of the subsequent formation of a structure consistent with that proposed by San George and Hoberman (22) at the α-amino terminus of the peptide. When San George and Hoberman generated acetaldehyde adducts of hemoglobin peptides and other model peptides, they used longer incubation periods (72 h) than we used in our experiments (1 h). In addition, they used higher concentrations of reactants for incubation (up to 100 mM acetaldehyde with 6 mM peptide) than were used in the present study. San George and Hoberman noted in their proposed mechanism for formation of the detected imidazolidinone adducts that a Schiff base intermediate product would be formed. The absence of Schiff base adducts in the final reaction mixture after purification would lead to the conclusion that any Schiff base structures formed were rapidly converted into the imidazolidinone derivatives. In addition, the high concentration (45 mM) of acetaldehyde used might have generated cross-linking in the globin chains during the incubation. In the generation of adducts for the present study, samples were exposed to 37 °C temperatures only for the length of incubation

(1 h). After incubation, the sample was stored at 4 °C prior to spectroscopic analysis.

The stability of the Schiff base acetaldehyde-pentalysine adducts suggests that some of the deleterious effects of acetaldehyde after ethanol consumption may be linked to stable Schiff base alterations in the structures of biologically important molecules. These structural changes may in turn affect the conformational properties of the protein or other bioactive molecules, thereby disrupting function *in vivo*.

The biological picture of acetaldehyde-protein adducts is a complicated one. Although the existence of stable Schiff base acetaldehyde-lysine adducts has been established here, it is not expected that these are the only adducts formed *in vivo*. Therefore, we plan to extend our analysis to blood proteins such as hemoglobin and albumin in order to determine the structural alterations of these molecules after reaction with acetaldehyde.

The demonstration of Schiff base adducts with lysine and acetaldehyde supports the use of the CHD-based fluorimetric assay as a measure of quantifying drinking behavior. The conditions of the assay (low pH, 70 °C) would be expected to facilitate the CHD reaction with Schiff base-derived acetaldehyde in whole blood. The acetaldehyde measured in our assay must be either free in the blood or it must be labile enough to be liberated during the assay. Therefore, it is less likely that we are measuring acetaldehyde from such structures as the imidazolidinone, although these structures may in fact be present in the blood sample. The CHD fluorimetric assay has proven useful in discriminating between ethanol-consuming and -nonconsuming mice, pigs, and humans (32).

In summary, our observations documenting that relatively stable Schiff base adducts occur following incubation of acetaldehyde with pentalysine *in vitro* are consistent with site and concentration specificity of these reactions. Further studies of larger molecules both *in vitro* and *in vivo* should provide insight into the molecular pathology of acetaldehyde-protein interactions.

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