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Binding of Acetaldehyde to a Glutathione Metabolite: Mass Spectrometric Characterization of an Acetaldehyde-Cysteinylglycine Conjugate

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Background: Ethanol administration decreases hepatic glutathione levels and increases urinary sulfhydryl excretion. Ethanol-induced liver injury is blunted by the administration of glutathione precursors. Acetaldehyde generated in the metabolism of ethanol binds to a number of amino acid residues in proteins and peptides, but it does not react readily with glutathione. Due to the possible role of acetaldehyde in cysteine and glutathione homeostasis, we investigated the reaction of acetaldehyde to cysteinylglycine, the dipeptide generated in vivo in the hydrolysis of glutathione by γ-glutamyltransferase.

Methods: A conjugate between acetaldehyde and cysteinylglycine was generated under physiologically relevant conditions, both in vitro and in vivo. It was separated by a new reverse-phase high-performance liquid chromatography method and identified by electrospray ionization/ion trap tandem mass spectrometric analysis.

Results: The conjugate with a stoichiometry of 1:1 between cysteinylglycine and acetaldehyde is most rapidly generated in vitro and was identified by mass spectroscopy as 2-methyl-thiazolidine-4-carbonyl-glycine. This thiazolidine derivative is stable in vitro and in biological fluids of rats. The conjugate was present in high concentrations in the bile of rats pretreated with ethanol and an inhibitor of aldehyde dehydrogenase.

Conclusions: The sequestering of cysteinylglycine by acetaldehyde occurs rapidly under physiologic conditions. Long-lived sulfur-containing biomolecules that incorporate acetaldehyde might affect cysteine and glutathione homeostasis and may also play a protective role by reducing circulating acetaldehyde levels. The acetaldehyde conjugate or its metabolic products could potentially serve as markers of ethanol consumption.

Key Words: Cysteinylglycine, Glutathione, Thiazolidine, Mass Spectrometry, Acetaldehyde.

ACETALDEHYDE, AN ETHANOL metabolite and toxic industrial chemical with high reactivity toward proteins, nonenzymatically forms conjugates in vitro (Braun et al., 1995; Donohue et al., 1983; Lin et al., 1995; Stevens et al., 1981); these are also detected in vivo in alcoholics (Niemela and Israel, 1992; Svegliati-Baroni et al., 1994). Free α-amino terminus, ϵ-amine side groups of lysine residues and sulfhydryl groups in proteins bind acetaldehyde (Braun et al., 1997; Koterba et al., 1995; Lin et al., 1995; San George and Hoberman, 1986). Protein-acetaldehyde conjugates set off the generation of antibodies against the new acetaldehyde epitopes (Israel et al., 1986; Klassen et al., 1999). Quantification of acetaldehyde-protein conjugates, directly or through their antibodies, has been used to evaluate chronic alcohol intake (Niemela and Israel, 1992; Viitala et al., 1997; Worrall et al., 1998). The acetaldehyde-induced antibodies have also been shown to be hepatotoxic in ethanol-fed animals (Yokoyama et al., 1995).

Both acute (Speisky et al., 1985) and chronic (Hirano et al., 1992; Lu et al., 1999) ethanol consumption reduce the levels of hepatic glutathione (GSH), a cysteine-containing tripeptide. Alcoholics with liver disease also show lower hepatic GSH levels (Videla et al., 1984). Supplementation with cysteine precursors reduces early-stage alcohol-induced liver injury both in humans (Mato et al., 1999) and experimental animals (Iimuro et al., 2000). GSH is a powerful antioxidant that is able to scavenge free radicals and hydrogen peroxide and to condense both enzymatically and nonenzymatically with toxic metabolites (Meister, 1995), facilitating their elimination. GSH was initially considered a plausible target for reactive acetaldehyde because both metabolic products are present at their maximum levels in

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the liver—GSH in the order of 5 mM and acetaldehyde up to 50 μM (Nuutinen et al., 1984). Further, both ethanol and acetaldehyde administered to rats lead to loss of thiols into urine (Hemminki, 1982). Nevertheless, several in vitro studies have indicated that GSH is relatively inert in forming conjugates with acetaldehyde (Cederbaum and Rubin, 1975; Kera et al., 1985; Speisky et al., 1985).

It was found initially that prolonged in vitro incubation of ^{14}C-acetaldehyde with ^{3}H-(Cys)-GSH led to the generation of a new product that carried both ^{14}C and ^{3}H (Anni et al., 2000). However, subsequent work pointed out that the conjugating partner to acetaldehyde is the dipeptide cysteinylglycine (CysGly), derived from the spontaneous nonenzymatic hydrolysis of GSH, rather than GSH itself (Anni et al., 2001). In vivo, CysGly is the first metabolic product of GSH hydrolysis by the ectoenzyme γ-glutamyltransferase (GGT) (Meister, 1995). In the liver, GGT is present mainly in the bile canaliculus (Lanca and Israel, 1991), where GSH concentrations can reach 4 to 7 mM (Ballatori et al., 1986).

We report the rapid in vitro conjugation of synthetic CysGly with acetaldehyde, the marked pH stability of the conjugate, and the determination of its structure by mass spectrometry. A thiazolidine derivative, 2-methyl-thiazolidine-4-carbonyl-glycine (MTCG), was identified as the stable conjugation product of CysGly with acetaldehyde. This conjugate was also detected in rat bile after acute ethanol treatment, under conditions in which increased acetaldehyde levels are present in the liver. The combination of CysGly with acetaldehyde may have a role in reducing plasma acetaldehyde. In addition, MTCG or its metabolic products may be valuable as biomarkers of alcohol intake.

MATERIALS AND METHODS

Reagents

CysGly, GSH, and acetaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). Oxidized CysGly (Cys-bis-Gly) was obtained from Bachem (King of Prussia, PA), oxidized GSH was obtained from Calbiochem (La Jolla, CA), and ethanol was obtained from Pharmco (Brookfield, CT). Ketamine HCl was obtained from Fort Dodge Animal Health (Fort Dodge, IA), xylazine was obtained from Phoenix Pharmaceutical (St. Joseph, MO), and isoflurane was obtained from Abbott Laboratories (North Chicago, IL).

Preparation of Conjugate

For analytical measurements, a reaction mixture of the reactants (CysGly and acetaldehyde) in 10 mM potassium phosphate buffer, pH 7.4, was incubated at 37°C for different time periods. At the end of the incubation period, samples were frozen in liquid nitrogen and lyophilized for removing excess acetaldehyde and for concentrating. Samples were analyzed immediately after lyophilization or stored at −80°C. All measurements were performed in triplicate.

Chromatographic Separation of Conjugate

The conjugate was separated by a reverse-phase (RP) high-performance liquid chromatography (HPLC) method based on an earlier method for separation of thiols (Liu et al., 1996), with some modifications. An isocratic method was developed with a mobile phase of 7% methanol in 10 mM potassium phosphate buffer adjusted to pH 3.0. The separation was performed on a Beckman Instruments (Fullerton, CA) HPLC system equipped with an Ultrasphere ODS column (25 cm × 4.6 mm; 5 μm) and a 20-μl injection loop (Anni and Israel, 1999), and the absorbance was monitored at 200 nm (Huber and Edwards, 1989). Under these conditions, the conjugate had a retention time of 5.6 min. Standard calibration curves were developed for CysGly and Cys-bis-Gly, and the integrated area of the HPLC peaks under the curve was used for quantification. The identity of the peaks was confirmed by using the retention times of authentic compounds (CysGly, Cys-bis-Gly, Cys, and Gly) and sample spiking. The retention times of Cys and Gly were 3.0 and 2.9 min, respectively.

Structural Analysis of Conjugate

Chromatographically purified conjugate was exchanged with a volatile solvent appropriate for mass spectrometry by a desalting RP-HPLC method by using a linear gradient 0–5% B [A, 0.1% trifluoroacetic acid (TFA)/water v/v; B, 0.1% TFA/acetonitrile (AcN) v/v] in 15 min. Measurements were followed at 215 nm because of the high absorbance at 200 nm of TFA in AcN. Under these conditions, the conjugate eluted as a major peak at 12.0 min. The concentration and purity of the desalted conjugate were established by RP-HPLC (5.6-min peak). The conjugate was dissolved in 50% AcN/100 mM ammonium acetate, pH 6.5, to aid ionization and was introduced into the electrospray source by constant infusion at 5 μl/min.

Tandem mass spectrometry (MS^4) was performed by electrospray ionization and an ion trap mass analyzer by using an offline ThermoFinnigan LCQ Deca mass spectrometer with Xcalibur software (San Jose, CA). Data were collected in both positive and negative ionization modes. The spray voltage for the positive mode was 4.5 kV and for the negative mode was 4.0 kV. The sheath and auxiliary gas flow rates were set at 0.68 and 0 liters/min, respectively, and the capillary temperature was 250°C.

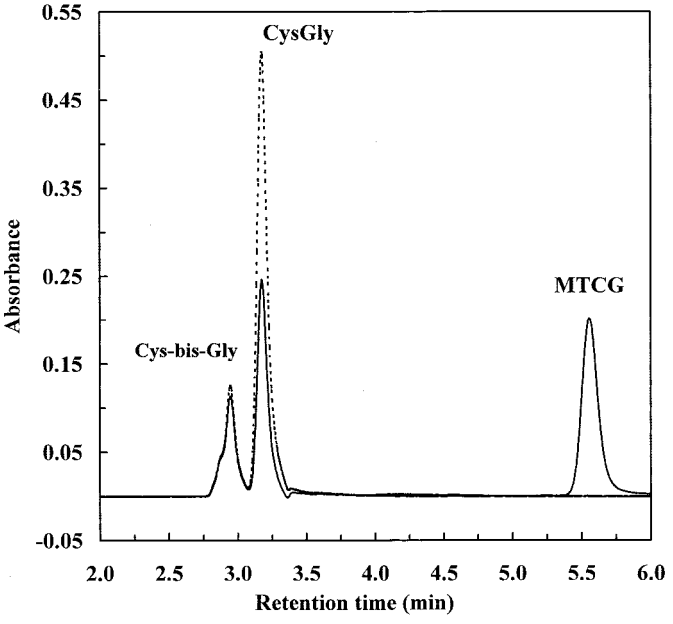

Fig. 1. Chromatographic separation of MTCG, a conjugate of CysGly with acetaldehyde. Representative RP-HPLC traces are shown of freshly prepared CysGly (0.5 mM) alone (dotted line) or with acetaldehyde (0.2 mM; solid line) after incubation in 10 mM potassium phosphate buffer, pH 7.4, at 37°C for 30 min. The 5.6-min peak was assigned to a conjugate, MTCG (see below). Freshly prepared CysGly preparations routinely contain approximately 16% Cys-bis-Gly (2.9-min peak), and under our experimental conditions, 8% more was produced. Absorbance was monitored at 200 nm. Other conditions are described in "Materials and Methods."

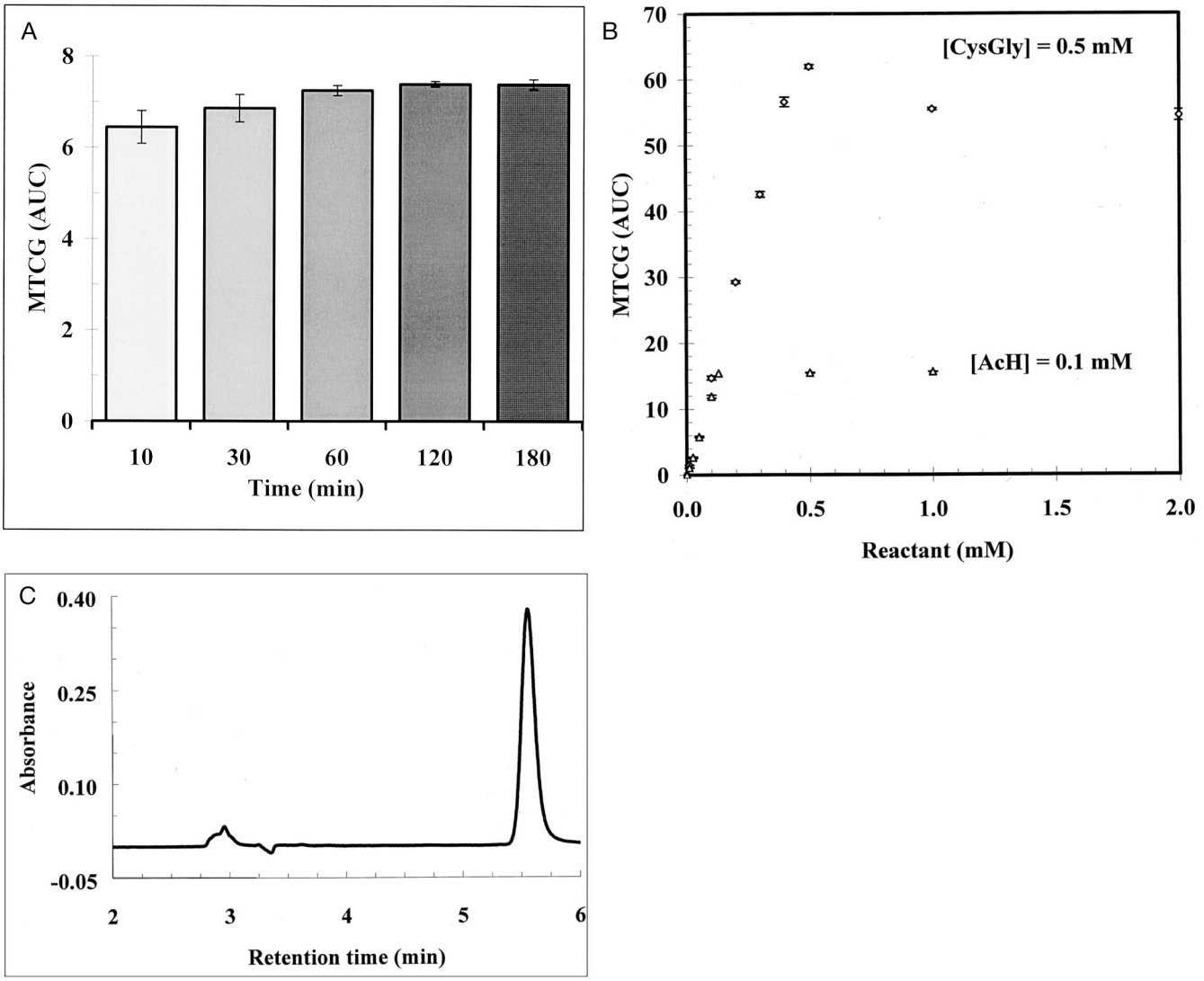

Fig. 2. Determination of parameters for conjugate production. (A) Time course of conjugation between CysGly (0.2 mM) and acetaldehyde (0.05 mM) in 10 mM potassium phosphate buffer, pH 7.4, at 37°C. The conjugate was separated by RP-HPLC, and its concentration is expressed as the integrated area under the curve (AUC). (B) Dependence of conjugate formation on CysGly concentration at a fixed concentration of acetaldehyde (0.1 mM; △) and on acetaldehyde (AcH) concentration at a fixed concentration of CysGly (0.5 mM; ◇) in 10 mM potassium phosphate buffer, pH 7.4, after incubation at 37°C for 30 min. (C) Chromatogram of CysGly (0.5 mM) incubated with excess acetaldehyde (1 mM).

Animals

Male rats were purchased from Harlan (Indianapolis, IN). Sprague-Dawley rats fed chow diet or Lewis rats fed control liquid diets, weighing 400–500 g, were used to determine the presence of MTCG in bile. Animals were fasted overnight and administered cyanamide (0.5 mmol/kg body weight, intraperitoneally), an inhibitor of aldehyde dehydrogenase. Three hours later, animals were administered 0.5 g/kg ethanol intravenously as a 20% v/v solution. Both cyanamide and ethanol were administered in saline. A group of control animals without cyanamide treatment was also tested. Ketamine (100 mg/kg) and xylazine (20 mg/kg) were administered intraperitoneally for anesthesia. Collection of bile started before ethanol injection to serve as a control baseline sample for each animal and was continued for 1 hr. Bile was collected in 0.5 ml of 0.3 M perchloric acid at 4°C in 10-min fractions, and protein-free supernatant was obtained by centrifugation at 10,000 $\times$ *g* for 10 min at 4°C before RP-HPLC chromatography was performed. Blood was collected thereafter from the hepatic vein for determination of acetaldehyde in plasma. Acetaldehyde levels in the plasma were measured after derivatization by RP-HPLC as described previously (Garver et al., 2000; Lucas et al., 1986). Samples were immediately processed and measured or stored at −80°C. Biological fluids from untreated animals were spiked with purified MTCG, and RP-HPLC was performed to check for its stability. All experimental procedures were approved by the Animal Care and Use Committee of Thomas Jefferson University and were performed according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

RESULTS

Conjugation of CysGly With Acetaldehyde

The conjugation product of CysGly with acetaldehyde was separated by an RP-HPLC method with monitoring of absorbance at 200 nm. Figure 1 shows that the addi-

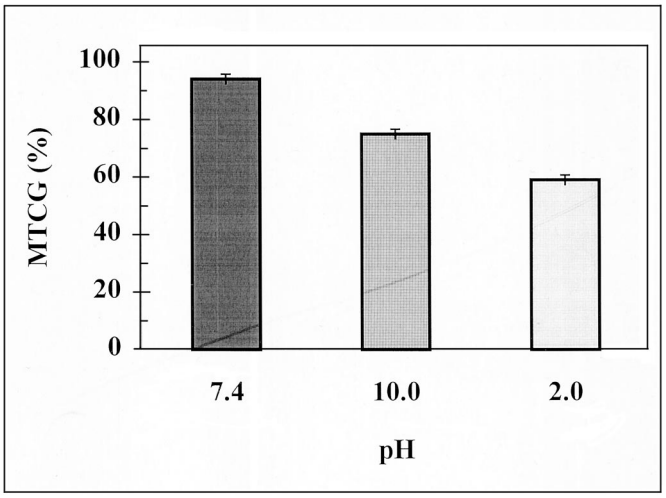

Fig. 3. Stability of preformed conjugate between acetaldehyde and CysGly as a function of pH. Stability is expressed as the recovery percentage of MTCG after a 1-hr incubation at 37°C at the indicated pH. Values are normalized, with 100% set for the amount of the preformed conjugate produced with 0.1 mM each of CysGly and acetaldehyde at pH 7.4 after incubation at 37°C for 30 min, before any pH treatment. Other conditions are described in the legend of Fig. 1.

tion of acetaldehyde to an excess amount of CysGly resulted in a decrease of the CysGly chromatographic peak (with a retention time of 3.2 min). Concomitantly, a peak with a retention time of 5.6 min appeared. The minor 2.9-min peak attributed to Cys-bis-Gly remained unchanged in the presence (solid line) or absence (dotted line) of acetaldehyde. The new 5.6-min peak MTCG was assigned to a conjugate between CysGly and acetaldehyde. Because acetaldehyde is known to conjugate Cys (Nagasawa et al., 1987), we tested whether the 5.6-min peak derived from Cys. Under the same experimental conditions, neither of the constituent amino acids of CysGly yielded the 5.6-min peak with acetaldehyde (data not shown). Likewise, the MTCG peak was absent when Cys-bis-Gly, lacking the free sulfhydryl group, replaced CysGly. All experiments reported were conducted at 10 mM phosphate buffer, at the pH indicated. There were no differences observed at 100 mM phosphate buffer (data not shown).

The conjugation of CysGly (0.2 mM) with acetaldehyde proceeded very fast, reaching 87% completion within 10 min (Fig. 2A) at concentrations of acetaldehyde (50 μM) that are found in the hepatic vein after consumption of alcohol (Nuutinen et al., 1984). All experiments described thereafter were performed by incubation of CysGly with acetaldehyde at 37°C for 30 min.

The stoichiometry of the reactants in the conjugate was established by varying the concentration of one reactant while keeping the other one constant (Fig. 2B). Data indicate that the amount of conjugate formed increased linearly with the concentration of the reactants until a 1:1 ratio was attained. The chromatogram of the reaction products between CysGly with excess acetaldehyde illustrates that all of the 3.2-min peak of CysGly is consumed and disappears (Fig. 2C). Taking into consideration the equimolar stoichiometry of the reactants in the conjugate and the amount of Cys-bis-Gly in the preparation, it was estimated that an average area under the curve of 14.3 corresponds to 0.1 mM of conjugate.

The conjugation reaction was practically independent of pH in the range of pH 5.0 to 9.0 (data not shown). The stability of the preformed conjugate (formed with 0.1 mM of each CysGly and acetaldehyde) was determined after 1 hr of incubation at 37°C under acidic and alkaline conditions. Data show that the conjugate was stable at neutral pH (94% recovery) but partially degraded at pH 10.0 and 2.0 (Fig. 3). The conjugate was also stable when added to biological fluids such as bile, plasma, and urine of naïve rats (data not shown).

Structural Analysis of Conjugate by Tandem Mass Spectroscopy

On the basis of 1:1 stoichiometry between acetaldehyde and CysGly in the conjugate (Fig. 2B) and the reactivity of acetaldehyde toward compounds carrying free sulfhydryl and amine groups (see "Discussion"), a conjugate was postulated with the structure of MTCG (Figs. 4A and 5A) and a molecular weight of 204. Mass analysis with positive and negative electrospray ionization/ion trap tandem mass spectroscopy was performed to confirm the hypothetical structure (Figs. 4 and 5). The ion trap collected batches of ions from the continuous ion current generated by the electrospray ionization source. First, the full scan of mass over charge (m/z) from 50 to 500 was recorded to detect a molecular ion with m/z of 205 in positive mode and 203 in negative mode. The intense molecular ions at m/z $(M + H)^+ = 205.1$ (Fig. 4A) and $(M - H)^- = 203.3$ (Fig. 5A) represent MTCG with positive and negative ionization, respectively. For MS measurements, the parent ion at m/z = 205.1 (Fig. 4A) was isolated by the ion trap, whereas all other ions were ejected. The parent ion *b* was then fragmented by applying additional energy in a collision-induced dissociation process to produce two daughter ions with m/z of 102.0 and 187.9 that were scanned out of the trap to the detector (Fig. 4B). Subsequently, one of these ions, *c*, with m/z = 187.9, was selected and further fragmented in MS^3, resulting in four main granddaughter ions at m/z of 56.0, 102.0, 116.0, and 159.8 (Fig. 4C). Finally, fragmentation of the most intense ion of this set (*d*) at m/z = 102.0 produced MS^4 with ions at m/z of 56.0 and 73.9 (Fig. 4D). Furthermore, fragmentation of the second daughter ion (*e*) at m/z = 102.0 from MS^2 yielded in MS^3 ions at m/z of 56.0, 73.9, and 85.1 (Fig. 4E). Similarly, in negative ionization mode, the m/z = 203.3 ion was fragmented to derive the ions with lower m/z, and the process was repeated four times, MS^4, until no more ions could be detected (Fig. 5A–D). The negative ionization data gave complementary information about the structure of the conjugate. The fragmen-

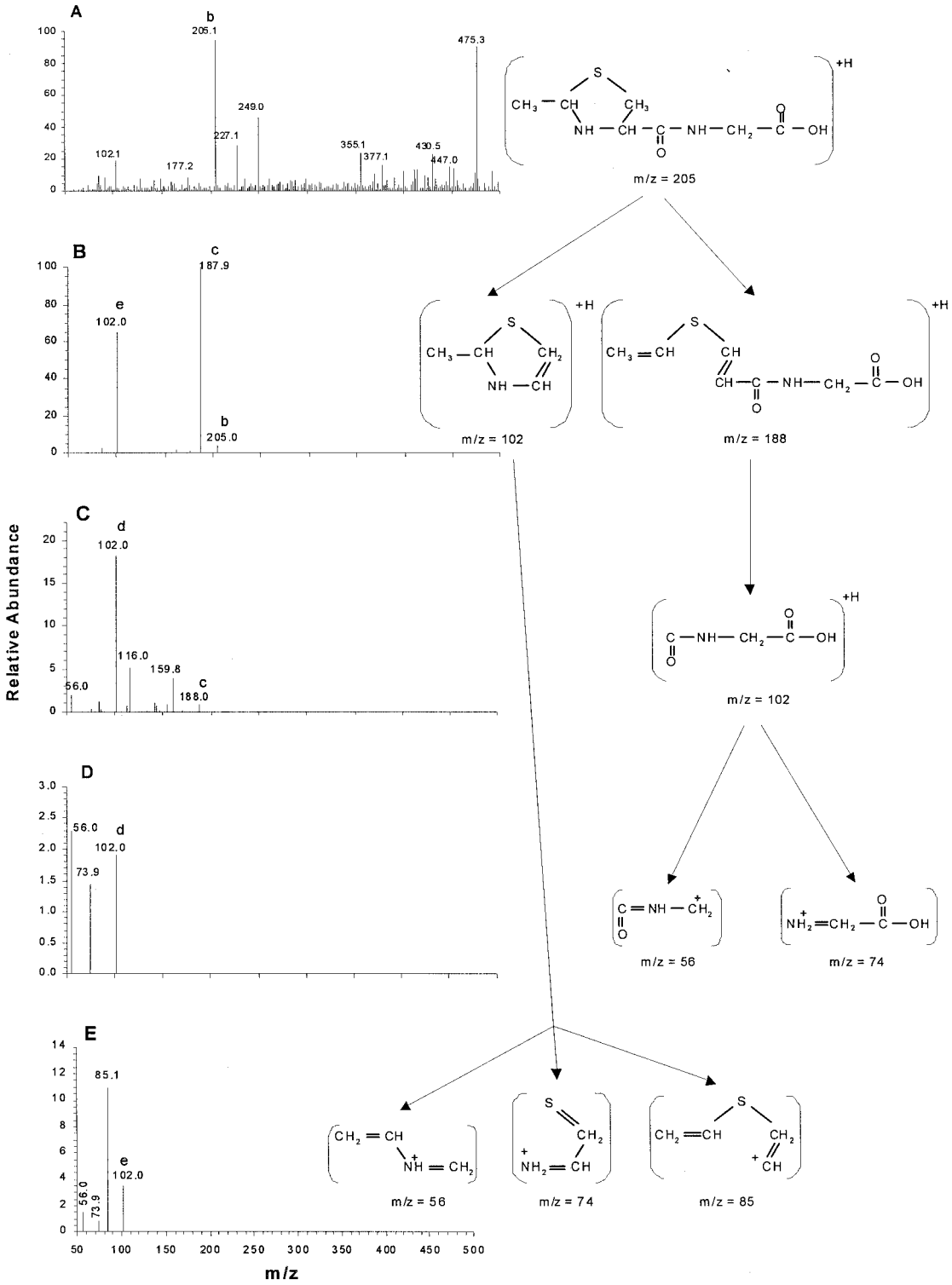

Fig. 4. Positive electrospray ionization/mass spectrometry (MS) of MTCG. (A) Full scan MS; (B) MS^2 fragmentation pattern of ion *b*; (C) MS^3 fragmentation pattern of ion *c*; (D) MS^4 fragmentation pattern of ion *d*; (E) MS^3 fragmentation pattern of ion *e*. Besides the parent ion, other ions seen in the full scan MS are likely impurities that ionize more readily than MTCG, like plasticizers in tubes used for sample preparation. Fragmentation conditions are presented in Table 1. Other conditions are described in "Materials and Methods."

tation patterns of MTCG with positive and negative ionization are summarized in Table 1. It is evident that the ions *d* and *e* with m/z of 102.0 represent different chemical entities that coincidentally have the same mass (compare Fig. 4B and 4C). This is also the case for the ions at m/z of 56.0 and 73.9 in Fig. 4D and 4E.

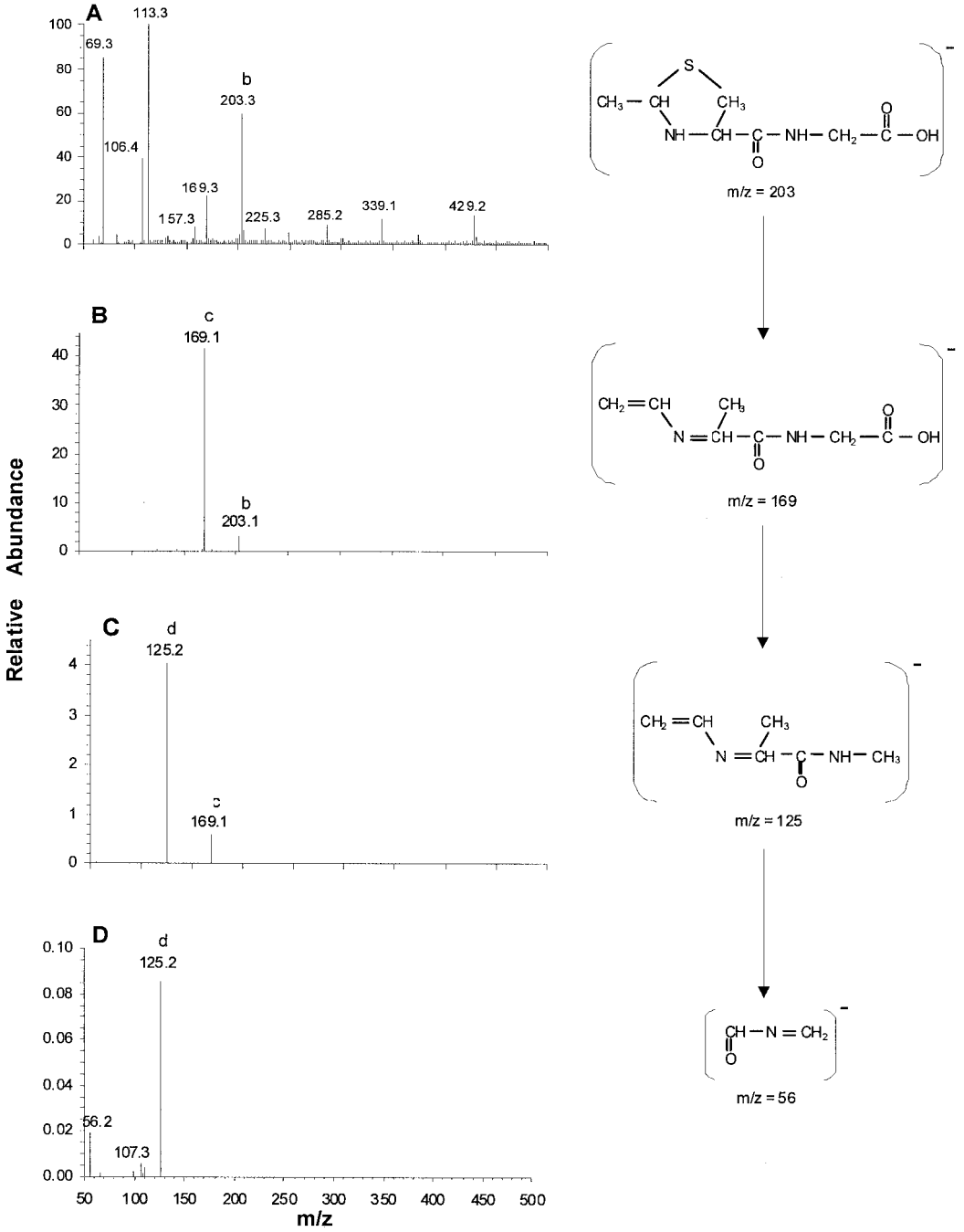

Fig. 5. Negative electrospray ionization/mass spectrometry (MS) of MTCG. (A) Full scan MS; (B) MS^2 fragmentation pattern of ion *b*; (C) MS^3 fragmentation pattern of ion *c*; (D) MS^4 fragmentation pattern of ion *d*. Fragmentation conditions are presented in Table 1. Other conditions are described in the legend of Fig. 4.

The conjugate, MTCG, was not detected in the bile of animals administered cyanamide alone while plasma acetaldehyde levels were minimally increased. Bile of animals ($n = 5$) treated with cyanamide and subsequently administered ethanol (0.5 g/kg) showed a large MTCG peak corresponding to 90 to 100 μM of the conjugate. Plasma acetaldehyde levels were also in the 100 to 120 μM range. In the absence of cyanamide, an acute ethanol dose does not produce a detectable MTCG peak.

DISCUSSION

The low reactivity of GSH with acetaldehyde reported in earlier studies (Cederbaum and Rubin, 1975; Speisky et al., 1985) led to a re-evaluation of the reaction by using ^{3}H-(Cys)-GSH and ^{14}C-acetaldehyde (Anni et al., 2000, 2001). A new compound with a discrete 5.6-min peak was detected by RP-HPLC after incubation for 24 hr in 10 mM potassium phosphate buffer, pH 7.4, at 37°C. Because the new compound contained both ^{3}H and ^{14}C, it was initially

Table 1. MS Fragmentation Pattern of MTCG With Positive and Negative Ionization

MS^1	MS^2	MS^3	MS^4
Positive ionization			
205.1	187.9	102.0	56.0
			53.9
	102.0	85.1	
		73.9	
		56.0	
Negative ionization			
203.3	169.1	125.2	56.0

All numbers represent m/z of positively or negatively ionized molecular ions detected in the mass spectra MS^1 to MS^4 of Figs. 4 and 5. For positive ionization mode, the normalized collision energy (NCE) used was 24, 22, and 23% for the series MS^1 to MS^4 and was 24 and 23% for the series MS^1 to MS^3, respectively. For negative ionization mode, NCE was 45, 51, and 53% for the MS^1 to MS^4 series. The NCE is a measure of the amplitude of the resonance excitation radiofrequency voltage applied to the endcaps of the ion trap that scales the amplitude of the voltage to the chosen ion mass. These values of NCE, expressed arbitrarily as % NCE, were chosen to fragment an ion to such an extent that a small amount of nonfragmented parent ion would still be detectable in the resulting MS spectra.

considered as a GSH-acetaldehyde conjugation product. Nevertheless, we subsequently established that during the extended incubation, some GSH was nonenzymatically hydrolyzed to CysGly. This suggested that the latter dipeptide, rather than GSH, might conjugate with acetaldehyde. The present studies assessed the nature of the conjugate formed between CysGly and acetaldehyde and its properties.

Structural Assignment of the Conjugate of CysGly With Acetaldehyde, and the Reaction Mechanism

We separated, by RP-HPLC, a new compound with a 5.6-min retention time that was generated in the reaction of acetaldehyde with CysGly under physiologically relevant concentrations: pH 7.4 and 37°C (Fig. 1). This conjugate is not generated from amino acids Cys or Gly in the presence of acetaldehyde (data not shown), although Cys conjugates acetaldehyde into 2-methyl-thiazolidine-4-carboxylic acid (Nagasawa et al., 1984). Compounds metabolically related to acetaldehyde—such as its precursor, ethanol (50 mM), and its metabolite, acetate (0.1 mM)—did not give rise to any new peaks on incubation with CysGly (0.5 mM; data not shown). Under our experimental conditions, the conjugate was formed readily (Fig. 2A) in a pH-independent reaction in the range of pH 5.0 to 9.0. The conjugation reaction was fast (nearly 90% was completed in 10 min) and virtually temperature independent at the range tested (4–37°C; data not shown). The dependence of conjugate formation on reactants concentrations exhibited a 1:1 stoichiometry of acetaldehyde to CysGly (Fig. 2B). In the proposed equimolar reaction between CysGly and acetaldehyde (Fig. 6), condensation of the two reactants into a Schiff base is followed by a cyclization reaction that yields a five-member ring compound, MTCG, with a molecular weight of 204. Structural analysis by MS^4 with positive ionization confirmed the conjugate as MTCG (Fig. 4). This assignment was also concurred by MS^4 data obtained with negative ionization (Fig. 5).

Our data are in agreement with those of an earlier report, in which loss of GSH sulfhydryl groups was observed after incubation with acetaldehyde on addition of GGT (Kera et al., 1985). The marked ability of CysGly, the hydrolytic product of GSH, to conjugate with acetaldehyde, in comparison to GSH, is conferred mainly by the availability of a free primary amine on the Cys moiety of CysGly, which in GSH is blocked by glutamate. As indicated previously, acetaldehyde can target both amino and sulfhydryl groups. Initially, acetaldehyde would form unstable Schiff bases, which can be further stabilized into a five-membered ring of imidazolidinone (San George and Hoberman, 1986). In CysGly, the vicinal substitution of cysteine with sulfhydryl and primary amino groups facilitates the formation with acetaldehyde of MTCG, a five-membered ring thiazolidine derivative. The reactivity of Cys-bis-Gly toward acetaldehyde is eliminated due to the lack of a free sulfhydryl group. Other structurally similar aminothiol compounds capable of conjugating nonenzymatically with acet-

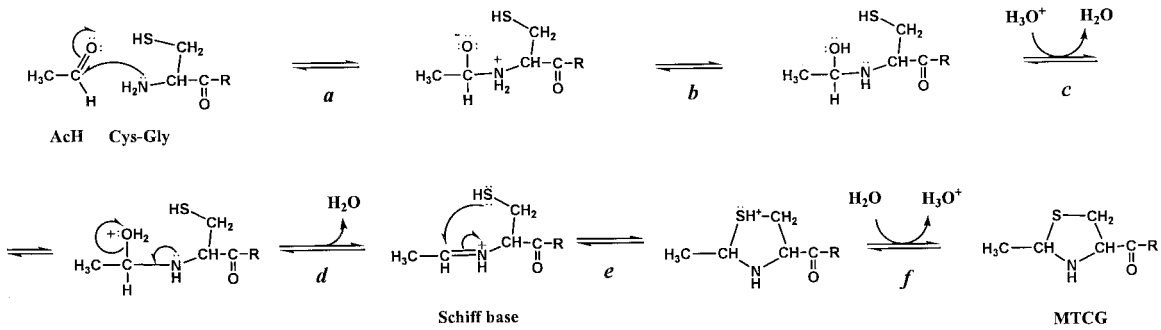

Fig. 6. Proposed mechanism of the reaction between CysGly and acetaldehyde to form a thiazolidine compound, MTCG. In reaction *a*, a dipolar tetrahedral intermediate is formed as a result of the nucleophilic attack of the lone pair electrons of the primary amine of CysGly on the carbonyl carbon of acetaldehyde. A proton is then transferred in reaction *b* from nitrogen to oxygen, yielding a neutral carbinolamine. An acid catalyst might protonate the carbinolamine oxygen to convert the hydroxyl into a better leaving group in reaction *c*. A protonated Schiff base is produced in reaction *d* with water expulsion by the nitrogen lone-pair electrons. In reaction *e*, the side-chain thiol adds across the double bond, closing the ring. Finally, loss of a proton from sulfur might regenerate the acid catalyst and give the stable five-member ring compound, MTCG. In the structure of CysGly, R stands for Gly.

aldehyde are the sulfhydryl amino acids homocysteine and penicillamine (Nagasawa et al., 1980).

Significance of Conjugate Formation Between Acetaldehyde and CysGly

In rats treated with disulfiram, an inhibitor of aldehyde dehydrogenase, the administration of D-penicillamine reduced the increased blood acetaldehyde levels after acute administration of ethanol (Nagasawa et al., 1980). Given the stability of MTCG in vitro at neutral pH and in biological fluids and the substantial recovery even under extreme pH conditions (Fig. 2C), the existence of MTCG in biological fluids was investigated. The conjugate was detected in the bile of rats administered ethanol acutely after pretreatment with cyanamide, an inhibitor of aldehyde dehydrogenase. The site of production of MTCG is most likely the bile itself, because CysGly is generated in the bile from GSH by canalicular GGT, and acetaldehyde is membrane permeable diffusing from hepatocytes, where it is produced by ethanol metabolism. Most of the 4 to 7 mM of GSH synthesized in rat liver (Ballatori et al., 1986) is hydrolyzed intrabiliary, with only 20% appearing in excreted bile (Sies, 1999). Moreover, chronic alcohol diet for 6 weeks leads to blood acetaldehyde levels of 50 μM in rats (Adachi et al., 1993). The concentration of MTCG in rat bile by RP-HPLC was determined to be 100 μM. Under our experimental setup, MTCG was not detected in rat plasma, although plasma acetaldehyde in the ethanol plus cyanamide–treated animals was approximately 100 μM. Hydrolysis of CysGly and the transport and reabsorption of specific biliary amino acids are processes that are not well understood. Because liver GGT activity is 7-fold higher in humans than rats (Hirai et al., 1994), high levels of CysGly from hydrolyzed GSH and, likely, MTCG, are expected in human bile. In human plasma, a 100 μM CysGly has been reported (Kleinman and Richie, 2000). In some reports (Kobayashi et al., 1983; Watanabe et al., 1985), blood acetaldehyde in some Japanese alcoholic patients can exceed 100 μM. Thus, CysGly may have a role in reducing blood and liver acetaldehyde levels.

Condensation of acetaldehyde with CysGly may explain the loss of hepatic Cys after the acute administration of ethanol (Speisky et al., 1985). Other studies indicated that acute administration of ethanol leads to the loss of sulfhydryl equivalents into the urine (Hemminki, 1982). Whether the formation of MTCG contributes, along with other mechanisms (Colell et al., 1997), to the overall decline of liver GSH levels that results from chronic ethanol intake (Lu et al., 1999) should be further studied.

In conclusion, our data demonstrate that (1) CysGly, a GSH-derived peptide, readily conjugates with acetaldehyde in vitro under physiologically relevant conditions to yield a thiazolidine derivative, MTCG, whose structure was confirmed by mass spectrometry; (2) preformed MTCG was stable at physiologic pH and in body fluids (plasma, bile, and urine) of rats; and (3) MTCG was detected in vivo in the bile of rats with increased acetaldehyde levels. Future studies could determine the metabolic fate of radioactive MTCG administered to animals, its possible role in the reduction in hepatic GSH levels after ethanol intake, and its potential as a marker of alcohol intake.

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