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RESEARCH ARTICLE

Acetaldehyde-derived modifications on cytosolic human carbonic anhydrases

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Abstract

Acetaldehyde can generate modifications in several proteins, such as carbonic anhydrase (CA) II. In this study, we extended *in vitro* investigations on acetaldehyde adduct formation by focusing on the other human cytosolic CA enzymes I, III, VII, and XIII. High-resolution mass spectrometric analysis indicated that acetaldehyde most efficiently formed covalent adducts with CA II and XIII. The binding of up to 19 acetaldehydes in CA II is probably attributable to the high number of lysine residues ($n = 24$) located mainly on the surface of the enzyme molecule. CA XIII formed more adducts (up to 25) than it contains lysine residues ($n = 16$) in its primary structure. Acetaldehyde treatment induced only minor changes in CA catalytic activity in most cases. The present study provides the first evidence that acetaldehyde can bind to several cytosolic CA isozymes. The functional consequences of such modifications will be further investigated *in vivo* by using animal models.

Keywords: Alcohol, acetaldehyde, adduct, mass spectrometry, modification

Introduction

Acetaldehyde, the first metabolite of ethanol, plays an important role in alcohol toxicity in several tissues because of its ability to form adducts with different proteins[1–4]. Stable or unstable covalent adduct formation between proteins and acetaldehyde leads to various changes in physicochemical properties of affected proteins[5–9]. These modifications have been previously reported *in vitro* and confirmed *in vivo* in alcohol abusers[6,7,10–12]. The adduct formation may lead to severe disturbances in normal cellular functions, which can contribute to several diseases linked to alcohol abuse[13–15]. In the process of adduct formation, lysine residues play a key role as the preferred amino acid in target proteins[16–23]. In fact, there are several proteins that have shown potential as targets for modification by acetaldehyde. These include, for example, haemoglobin, collagens, albumin, transferrin, tubulin, and lipoproteins[14,24–26].

In the present study, cytosolic carbonic anhydrase (CA) isozymes I, II, III, VII, and XIII were used as model enzymes to investigate the structural and functional consequences of acetaldehyde-derived modifications in enzyme molecules *in vitro*. CA I, CA II, and CA VII were selected as target proteins because they are highly expressed in erythrocytes (CA I and II) and hepatocytes (CA VII)[27,28]. Both erythrocytes and hepatocytes represent typical cells where alcohol abuse can cause morphological changes. Most of these isozymes are also well-characterized and expressed in several other human organs and tissues, such as the alimentary canal epithelium (CA I, II, and XIII), skeletal muscle (CA III), the lens of the eye (CA I), the brain (CA II and VII), the

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endometrium (CA XIII), and renal tubules (CA II and XIII)[27,29–35]. The recombinant CA enzymes were treated with different concentrations of acetaldehyde under non-reducing or reducing conditions. High-resolution electrospray ionization Fourier transform ion cyclotron resonance (ESI FT-ICR) mass spectrometry was then used to determine the covalent binding of acetaldehyde to each isozyme. In addition to the mass spectrometry-based structural studies, a kinetic stopped-flow activity assay was performed to monitor possible functional changes in CA isozymes, which could be attributed to acetaldehyde binding. Functional defects in these proteins caused by adduct formation could potentially lead to abnormal pH regulation and carbon dioxide removal in tissues of alcohol abusers.

Materials and methods

Production of recombinant human CA isozymes

The recombinant human CA II enzyme was produced in *Escherichia coli*[36] and purified to homogeneity using CA inhibitor affinity chromatography as previously described[37]. The recombinant human CA VII and XIII enzyme production and purification procedures have been previously described[27,38].

For human CA I and III enzymes, the complete coding sequences were obtained from GenBank (accession numbers for CA I BC027890 and for CA III BC004897). The *CA1* and *CA3* cDNAs were amplified by polymerase chain reaction (PCR) using primers designed based on published information on *CA1* and *CA3* mRNAs in GenBank (accession number BC027890 and BC004897 for CA III). To generate the glutathione *S*-transferase (GST)-CA I and GST-CA III constructs, the following primers were designed: the CA I forward primer was (*Bam*HI F) 5′-CGCGGATCCATGGCAAGTCCAGACT GGGGA-3′; the reverse primer was (*Xho*I R) 5′-CCG CTCGAGTCAAAATGAAGCTCTCACTGT-3′; and the predicted amplification product was 804 bp. The CA III forward primer was (*Bam*HI-F-HCA III) 5′-CGCGG ATCCATGGCCAAGGAGTGGGGCTAC-3′, the reverse primer was (*Xho*I-R-HCA III) 5′-CCGCTCGAGTTACTA TCATTTGAAGGAAGC-3′, and the predicted amplification product was 807 bp. The reagents used for the PCR were purchased from Fermentas GmbH (St. Leon-Rot, Germany). PCR experiments were performed on a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA). The protocol consisted of a 98°C denaturation step for 3 min followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR product was analysed using 1.5% agarose gel containing 0.1 mg/mL ethidium bromide. DNA standards (100 and 1000 bp DNA ladders) were obtained from New England Biolabs (Beverly, MA). The amplified cDNAs for both CA I and III were digested with *Bam*HI and *Xho*I (New England Biolabs) and ligated into the *Bam*HI–*Xho*I-digested pGEX-4T-1 vector (Invitrogen, Carlsbad, CA). This strategy positioned the CA cDNAs in contact with a DNA encoding GST; also, a cleavage site of thrombin protease between the GST and CA domains was included. The pGEX-4T-1/GST-CA I and pGEX-4T-1/GST-CAIII constructs were transformed into *E. coli* BL21 (DE3) (Invitrogen) for subsequent expression.

Single colonies of *E. coli* BL21 (DE3) transformants with the pGEX-4T-1/GST-CA I and pGEX-4T-1/GST-CA III constructs were grown in 10 mL culture overnight and then grown in 1 L of LB broth base containing 50 mg/mL ampicillin with shaking (250 rpm) at 37°C until the optical density (OD) at 600 nm reached 0.6. The expression of the CA I and III enzymes was optimized by induction using isopropyl-β-D-thiogalactopyranoside(IPTG)(Fermentas) with a final concentration of 1 mM at 37°C for 3 h. The cells were harvested by centrifugation at 5000 *g* for 7 min at 4°C. Human CA I protein was purified under native conditions using a Glutathione Sepharose™ column (GE Healthcare, Buckinghamshire, UK). Restriction-grade, site-specific thrombin (GE Healthcare) (15 μL) was used for specific cleavage of the GST by overnight shaking at room temperature (RT).

For CA III protein purification, the cell pellet was suspended in 25 mL of Tris-buffer containing 0.1 M Tris–HCl, pH 7.0, 0.1% Triton X-100, 0.45 g lysozyme, 1 tablet of protease inhibitors (Complete Protease Inhibitor Cocktail Tablets™), and 20 mL DNase I (Roche, Basel, Switzerland). The *E. coli* cell suspension was kept on ice while lysed and sonicated for 3 min. The suspension was clarified by centrifugation at 12,900 *g* for 15 min at 4°C and the clear supernatant was used for affinity purification on Glutathione Sepharose™ column (GE Healthcare). CA III recombinant protein was isolated under native conditions, and a restriction-grade, site-specific thrombin (GE Healthcare) (15 μL) was used for specific cleavage of the GST by overnight shaking at RT. After treatment with thrombin, the target protein was eluted using glutathione according to the manufacturer's instructions (GE Healthcare).

The purity of the CA isozymes was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis performed under reducing conditions[39]. We also confirmed that each polypeptide band represented the correct protein by matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) analysis, which was performed with an Autoflex III mass spectrometer (Bruker Daltonik, GmbH, Bremen, Germany) equipped with a SmartBeam™ laser (355 nm). Protein identification was performed by searching the peptide monoisotopic masses for peptide mass fingerprints or the amino acid sequence tag for peptide fragments in MS/MS against the NCBInr database using Matrix Science's Mascot (http://www.matrixscience.com/, Matrix Science Ltd., London, UK). FlexAnalysis™ and Biotools™ software (Bruker Daltonics, Billerica, MA) were used to analyse MS data and as a search engine interface between raw

data transfer and the databases in the Mascot server, respectively. MALDI-TOF and protein identification were performed at the Proteomics Core Facility of the University of Helsinki.

Acetaldehyde treatment of CA I, II, III, VII, and XIII

Human recombinant cytosolic CA enzymes were treated with various concentrations of acetaldehyde either in the presence or absence of the reducing agent, $NaBH_3(CN)$. The experiments for CA II have been described in our recent work[40]. To minimize the evaporation of acetaldehyde, all reagents were kept on ice or at 4°C and pipetting was performed at 4°C. The sample tubes containing CA enzymes, with or without acetaldehyde, were tightly sealed and incubated at 37°C for 2 h. Then, either 10 mM $NaBH_3(CN)$ (for reducing conditions) or equal volume of H_2O (for non-reducing conditions) was added to each sample tube and incubated at 37°C for 22 h. After the incubation, the samples were quickly cooled down to 4°C.

Mass spectrometry

For mass spectrometry, protein samples were desalted as previously described[27]. For the desalted samples, protein concentrations were determined by absorbance at 280 nm using sequence-derived extinction coefficients. All experiments were performed on a 4.7-T Bruker APEX-Qe FT-ICR mass spectrometer (Bruker Daltonics), which was interfaced to an external Apollo-II ESI source. Protein samples were directly infused at a flow rate of 1.5 µL/min. ESI-generated ions were externally accumulated in an RF-hexapole ion trap for 500 ms and transmitted through a high-voltage ion optics region to an Infinity ICR cell for 'sidekick' trapping, conventional 'RF-chirp' excitation and broadband detection. For each spectrum, a total of 128–1000 co-added (512-kWord) time-domain transients were zero-filled once, prior to fast Fourier transformation and magnitude calculation. Frequency-to-m/z calibration was performed externally with respect to the ions of an ES Tuning Mix (Agilent Technologies, Santa Clara, CA) calibration mixture. All data were processed and analysed using Bruker XMASS™ version 7.0.8 software.

CA activity measurement

An Applied Photophysics stopped-flow instrument was used to assay CA-catalysed CO_2 hydration activity[41]. Reactions were measured using 0.2 mM phenol red (Abs_{max} at 557 nm) as the indicator, in 10 mM HEPES, 0.1 M Na_2SO_4, pH 7.5, for a period of 10–100 s. To determine the kinetic parameters and inhibition constants, the CO_2 concentration ranged from 1.7 to 17 mM. For the inhibitor assay, at least six traces of the initial reaction were used for determining the initial velocity, which were subtracted from the uncatalysed reaction. A stock solution of 1 mM acetazolamide in 10–20% (v/v) dimethyl sulphoxide (DMSO) was used to prepare dilutions up to 0.01 nM. Inhibitor and enzyme solutions were pre-incubated prior to inhibition measurements for 15 min at 25°C to form the enzyme–inhibitor complex. Inhibition constants were obtained using the non-linear least-squares methods using PRISM 3 and represent the mean from at least three different determinations, whereas kinetic parameters were obtained from Lineweaver–Burk plots, as reported earlier.

Detection of autoantibodies against CA enzymes

Serum samples for autoantibody assays were obtained from abstainers ($n = 16$, including 10 men, 6 women, mean age 44 ± 9 years) and alcoholic subjects ($n = 32$, including 28 men, 4 women, mean age 46 ± 10 years). The alcoholic patients were all subjects who had been consecutively admitted for detoxification with a history of continuous ethanol consumption or binge drinking, characterized by consumption of over 80 g/day during the 4 week-period prior to sampling. All participants gave their informed consent, ethical permission was obtained from the Ethics Committee of the EP Central Hospital, and the study was conducted according to the provisions of the Declaration of Helsinki.

Autoantibody levels were determined using an enzyme-linked immunosorbent assay (ELISA) developed in our laboratory. The protocol for the immunoassay included the following steps: purified CA I, II, and VII proteins (50 ng/well) in sodium carbonate buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6) were incubated on 96-well plates (Thermo Scientific Pierce, Waltham, MA) overnight at 4°C. The wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and blocked with 2% bovine serum albumin/PBST for 2 h at RT. After being washed three times with PBST, serum samples were diluted (1:5000, v/v) in 2% bovine serum albumin/PBST and incubated (50 µL/well) overnight at 4°C. Each sample was analysed in duplicate. After three washings with PBST, diluted (1:25,000, v/v) peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added (50 µL/well) and incubated for 1 h at RT. The plates were washed three times with PBST and incubated with a TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Thermo Scientific Pierce) for 15 min. The reaction was stopped with 2 M H_2SO_4 and the absorbance was measured at 450 nm. Serum samples from two healthy subjects (male and female) served as controls to estimate the baseline level of absorbance. The relative autoantibody levels were determined by dividing the mean absorbance levels of each sample (measured in triplicate) by the mean absorbance of the control samples. Anti-CA antibody levels were handled as continuous variables, and an unpaired *t*-test was used for comparisons between the groups. The findings were considered statistically significant at $P < 0.05$. The statistical analyses were performed using the GraphPad Prism™ version 5.01 software.

Results

Production, purification, and characterization of recombinant human CA I, II, III, VII, and XIII enzymes

The production and purification of human CA II, VII, and XIII have been described in previous articles[27,38,40]. The other cytosolic isozymes, CA I and III, were produced to conduct the present investigations. The human CA I and III cDNAs were cloned into the expression vector, pGEX-4T-1, and expressed as fusion proteins with GST. Figure 1 shows an SDS-PAGE analysis of all the recombinant proteins used in the present study. Upon digestion with thrombin and purification by affinity chromatography, all isozymes appeared as single 28–30 kDa polypeptides in the SDS-PAGE, as expected. The polypeptide bands were isolated from the gel and confirmed to represent the correct proteins by MALDI-TOF mass spectrometry (data not shown) prior to the experiments with acetaldehyde.

Mass spectrometry of CA proteins treated with acetaldehyde

High-resolution ESI FT-ICR mass spectrometry was used to detect acetaldehyde-derived covalent modifications in the produced recombinant CA isozymes. The protein samples were treated with acetaldehyde either in the presence or absence of a reducing agent, $NaBH_3(CN)$, to induce chemical modifications; the analysis was performed using mass spectrometry. The reactions were performed at the constant CA concentration of 100 μM and the acetaldehyde concentrations were increased to 200 mM (equal to molar ratios of 1:10 to 1:2000); afterwards, the reaction mixtures were diluted with acetonitrile/water/acetic acid (49.5:49.5:1.0, v/v) to a CA concentration of 5–10 μM prior to the measurement. The achieved high-resolution mass spectra enabled unambiguous detection and identification of different unstable and stable modifications. Molecular masses were first determined for native proteins (not treated with acetaldehyde) and were in good agreement with the theoretical masses calculated from the protein sequences (Table S1).

In previous studies with CA II isozyme, under non-reducing conditions only a single, but highly specific (>90%), unstable aldehyde adduct (a Schiff base; +26 Da) was formed, most likely with one of the 24 lysine residues in CA II[40]. In contrast, under reducing conditions a number of stable aldehyde adducts were formed (up to 19 adducts were detected). A Schiff base (imine) formation between the ε-NH_2 (side chain amino group) of lysine and acetaldehyde is highly pH-dependent and reversible (a rehydration of imine provides a free amine and aldehyde), which makes these adducts unstable and difficult to detect. Under reducing conditions, however, the imine is rapidly and irreversibly converted to the corresponding amine (*N*-ethyl lysine; +28 Da), which is a stable covalent adduct.

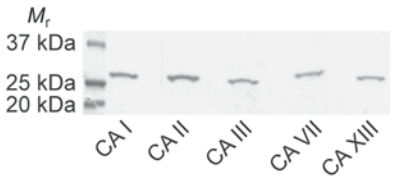

Figure 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of the recombinant human carbonic anhydrase (CA) isozymes used for the present analyses.

Figure 2 shows the mass spectra for native and acetaldehyde-treated CA I. Under non-reducing conditions, only very modest adduct formation was observed, even at the highest acetaldehyde concentrations (100–200 mM). Interestingly, two different adducts were formed, those consistent with the Schiff base formation showed a mass increment of 26 Da, whereas the other displayed a mass increment of 44 Da, most likely representing an unstable carbinolamine intermediate (primary amine–aldehyde addition product from which an imine is formed by dehydration). In contrast, under reducing conditions, a number (3–4 on average) of stable *N*-ethyl lysine residues (+28 Da) were already formed at 1 mM acetaldehyde concentration and a considerably increasing number (13–14 on average) were formed at 10 mM, which is more or less the same as observed with CA II[40].

Similar results were obtained for the other CA isozymes (data presented as Supporting Material; Figures S1–S3), with some minor differences. CA VII did not form any detectable adducts under non-reducing conditions, even at the highest aldehyde concentrations (Figure S2). In reducing conditions, a number of stable adducts ($n \times +28$ Da) were formed (6–8 on average with 10 mM acetaldehyde), which was a lower number than observed for CA I and II. A modest adduct formation (+26 and +44 Da adducts) was also observed for CA XIII in non-reducing conditions, but only at the highest acetaldehyde concentration (Figure S3). Similar to the other isozymes, many more adducts were formed in the reducing conditions, but decreasing spectral resolution with the highest aldehyde concentrations did not permit the accurate quantification of adducts formed. Nevertheless, inspection of the mass spectra indicated that the adduct formation showed a bimodal character with up to 25 adducts formed with 10 mM acetaldehyde. This was an interesting finding because this number clearly exceeded the number of lysines present in the structure (there are 16 lysines in CA XIII). This was not observed with any other isozyme (there are 18, 24, 18, and 13 lysines in CA I, II, III, and CA VII, respectively). It is notable that all the lysine residues are exposed on the surface of CA enzymes. The fewest adducts were formed with CA III (Figure S1). Only one unstable (+26 Da) adduct was detected at low levels with 100 mM acetaldehyde in non-reducing conditions but considerably more (roughly 5–7 stable +28 Da adducts) in reducing conditions. Again, the spectral resolution decreased and did not permit accurate quantification of the adducts formed with this protein.

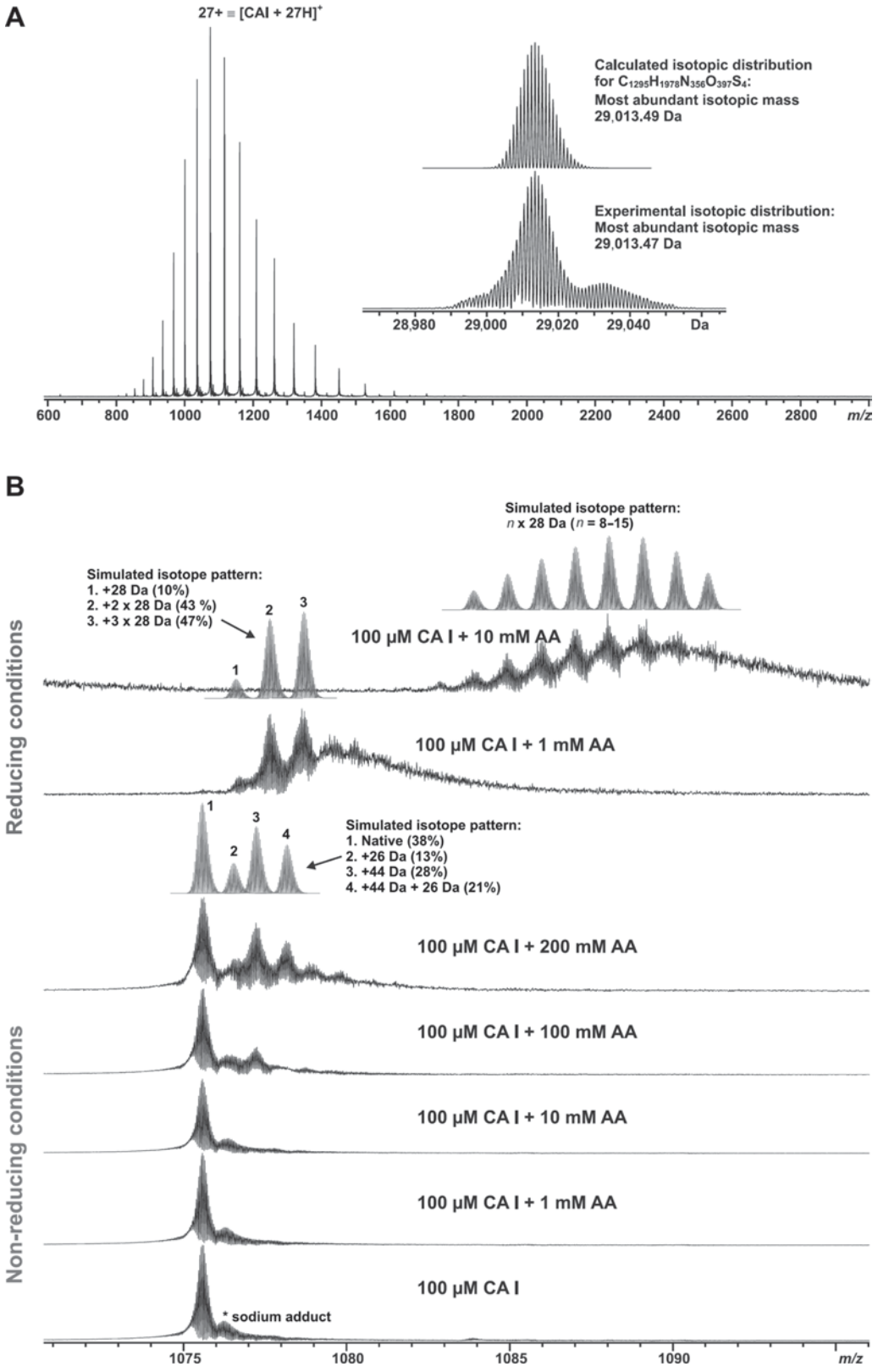

Figure 2. Electrospray ionization Fourier transform ion cyclotron resonance (ESI FT-ICR) mass spectra of recombinant human carbonic anhydrase (CA) I measured in acetonitrile/water/acetic acid (49.5:49.5:1.0, v/v) solvent. (A) A broadband mass spectrum of native CA I (protein ion charge state 27+ labelled) and the corresponding charge-deconvoluted mass spectrum (inset), showing both experimental and calculated isotopic distributions. (B) Partial mass spectra (expanded view on the charge state 27+) for acetaldehyde-treated CA I. An adduct with a mass increment of +26 Da corresponds to a Schiff base (imine) formation between acetaldehyde and a free amino group (lysine side chain) in the protein, and an adduct of +44 Da corresponds to a carbinolamine intermediate (see text for details). The simulated isotopic patterns were calculated from the elemental formulae of different protein forms using a custom Tcl-script embedded in XMASS™ software. A non-specific sodium adduct (+22 Da), resulting from incomplete protein desalting, is marked with a star.

Kinetic analysis

The effects of the formed acetaldehyde-derived modifications towards catalytic activity were analysed by a stopped-flow CA activity assay (Table 1). Recently published data indicate that the activity of CA II was significantly decreased in the presence of 100 mM acetaldehyde[40]. Under reducing conditions, the k_{cat}/K_M values for native and acetaldehyde-treated CA II enzyme were 1.5×10^8 and 0.5×10^8 $M^{-1}s^{-1}$, respectively. CA VII was considered an important enzyme candidate for acetaldehyde-derived modifications because of its high expression levels in hepatocytes[27]. Low physiological acetaldehyde concentrations (up to 1 mM) had virtually no effect on CA VII activity nor did they change the K_I value for acetazolamide (Table 1). Investigations also involved the other cytosolic enzymes, CA I, III, and XIII. Even the supraphysiological acetaldehyde concentrations ranging from 9 to 500 mM showed only modest effects on activity levels. Among these isozymes, CA XIII showed the highest number of changes, although they were still fewer than those observed for CA II (Table 1). Interestingly, small changes observed in the k_{cat}/K_M values resulted from changes in the catalytic turnover (k_{cat}) because the K_M values were virtually the same in each case. In addition, for all isozymes, except for CA VII, K_I values for acetazolamide were generally higher at higher acetaldehyde concentrations.

Table 1. Kinetic and inhibitory properties of human carbonic anhydrase (CA) isozymes measured by the stopped-flow method.

Enzyme	AA (mM)	K_M (mM)	k_{cat}/K_M ($M^{-1}s^{-1}$)	K_I (AAZ) (nM)
CA II	0	9.3	1.5×10^8	12
CA II + BH	100	9.3	0.5×10^8	35
CA I	0	4.0	4.93×10^7	260
CA I + BH	0	4.1	3.40×10^7	1734
CA I	9	4.0	3.86×10^7	1575
CA I	50	4.1	3.81×10^7	1613
CA I	500	4.2	3.80×10^7	1654
CA I + BH	50	4.0	3.37×10^7	1642
CA III	0	52.0	2.53×10^5	240
CA III + BH	0	52.5	2.21×10^5	534
CA III	9	52.3	2.36×10^5	254
CA III	50	52.1	2.34×10^5	437
CA III	500	52.0	2.18×10^5	510
CA III + BH	50	52.1	2.15×10^5	537
CA XIII	0	14.0	1.12×10^7	16
CA XIII + BH	0	14.1	1.12×10^7	164
CA XIII	9	14.0	1.05×10^7	21
CA XIII	50	14.2	0.84×10^7	163
CA XIII	500	14.3	0.81×10^7	174
CA XIII + BH	50	14.3	0.80×10^7	179
CA VII	0	11.4	8.3×10^7	2.5
CA VII + BH	0	11.4	8.4×10^7	2.3
CA VII	1	11.4	7.8×10^7	2.4
CA VII + BH	1	11.5	6.0×10^7	2.5

Data on CA II has been published previously[41].
AA = acetaldehyde, AAZ = acetazolamide, BH = $NaBH_3(CN)$.

CA autoantibodies

The possible anti-CA I, -CA II, and -CA VII autoantibody responses in alcoholic subjects were analysed using recently developed immunoassays for each of these autoantibodies and the results were compared with the values obtained from non-alcoholic subjects. The mean absorbance levels did not differ significantly between the abstainer and alcoholic subjects, suggesting that alcohol abusers show no detectable IgG class autoantibody response against these CA isozymes (Figure 3).

Discussion

In the present study, investigations of acetaldehyde-derived chemical modifications were extended to all cytosolic CA enzymes. They represented potential physiological targets for such modifications because these enzymes are highly expressed in cells, such as hepatocytes, skeletal muscle cells, and erythrocytes, where acetaldehyde–protein adducts have been previously located[2,4,10,42]. Importantly, they contain relatively high number of lysine residues on the surface of the proteins that were considered primary targets for aldehyde adduct formation.

Based on the results presented here and in previous work[40], there were clear differences among the studied CA isozymes to form acetaldehyde adducts in both non-reducing and reducing conditions. The mass spectrometry data indicated that in non-reducing conditions, all CA isozymes, except for CA VII, form unstable adducts (Schiff base) only at supraphysiological acetaldehyde concentrations (100 mM or higher). This was not surprising given that a Schiff base (imine) is relatively unstable and can easily be rehydrated to provide a free amine and aldehyde via a carbinolamine intermediate. The same has also been observed with covalent adducts formed upon the treatment of polypeptides with formaldehyde[43]. In contrast, all CA isozymes formed a number of stable covalent adducts (presumably *N*-ethyl lysines) in reducing conditions with some differences. It is likely that the solvent-exposed lysine residues were the primary targets for the aldehyde adduct formation, whereas some other residues could also form adducts[43]. In recent studies with small model peptides, arginine and cysteine residues were observed to be capable of forming adducts with acetaldehyde, particularly in reducing conditions (Jänis J. et al., unpublished results).

The most interesting results came from CA II and XIII; CA II formed a highly specific (>90%) single adduct in non-reducing conditions, whereas CA XIII was capable of forming more adducts in reducing conditions compared with the amount of lysine residues it contained in its primary structure. This observation suggests that some residues of this particular isozyme, other than lysines, are capable of forming stable adducts, the most probable being arginine residues. However, further identification and localization of these modifications may be experimentally difficult, given the number of adducts formed.

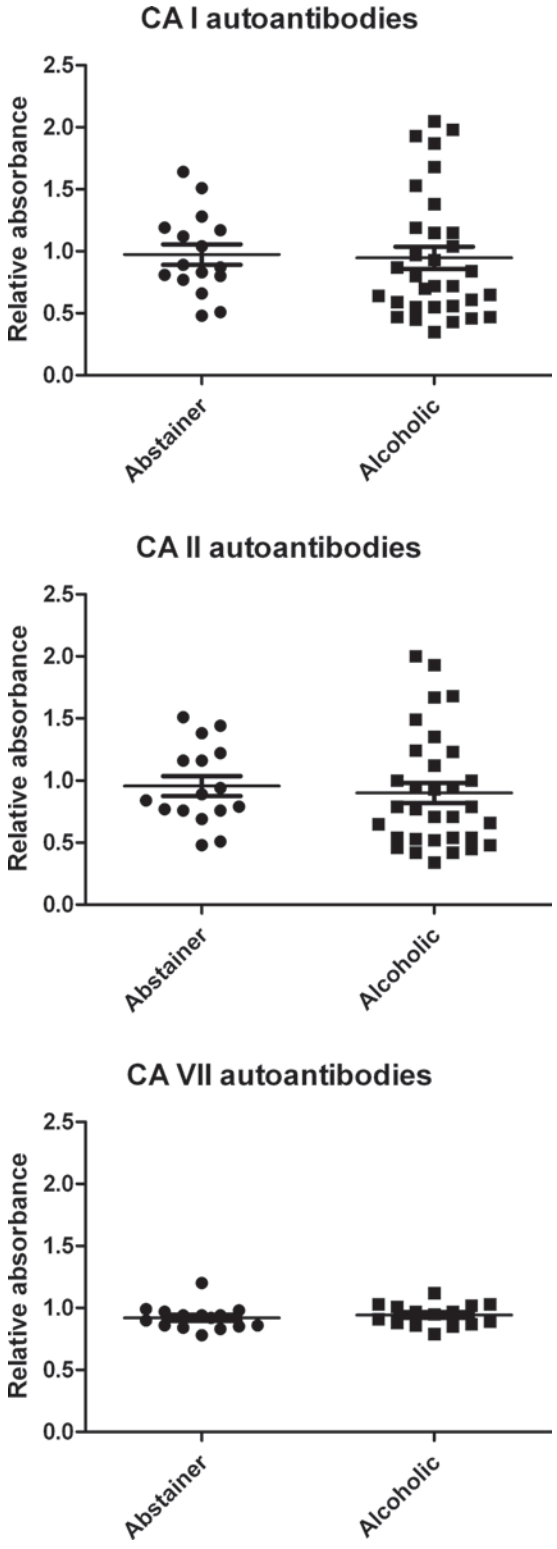

Figure 3. IgG class autoantibody levels for carbonic anhydrase (CA) I, II, and CA VII isozymes in abstainer and alcoholic groups. The mean antibody levels were almost identical between these two groups.

A structural comparison of different CA isozymes was made to investigate whether the propensity for the formation of adducts, especially in reducing conditions, was attributable to the number of surface exposed lysine (or other modifiable) residues. Crystal structures of the studied CA isozymes revealed that all the lysine residues reside on the protein surface (data not shown); thus, they were readily available for adduct formation, depending on solution conditions. Therefore, it was difficult to judge why different CA isozymes displayed differences in their adduct formation, as revealed by mass spectrometry, on the basis of the published three-dimensional structures. Since most lysine residues reside on the surface of the enzyme molecule and none of them are located in the active site cavity near the zinc atom (except for Lys64 in CA III), it is unlikely that these modifications have profound effects on the kinetic properties of cytosolic CA isozymes. This was supported by the present kinetic analyses, which showed only minor changes in the enzyme activity levels. Even though the enzyme activities almost remained at the same level, it is possible that acetaldehyde modifications can lead to more profound changes in cellular environment. At least some CA isozymes execute their function in metabolons with bicarbonate transporters and use surface structures for the protein–protein interactions[44]. Thus, acetaldehyde-derived modifications may affect the enzymatic performance of CAs in the metabolons. Interestingly, the inhibition constant for acetazolamide was markedly reduced in CA I and XIII after the acetaldehyde treatment or after the $NaBH_3(CN)$ treatment, even without acetaldehyde. It is possible that these compounds may act as weak inhibitors, interfering with the binding of the sulphonamide to the metal ion or to its accommodation within the restricted space of the active site.

Structural changes in proteins due to acetaldehyde binding have been shown to stimulate immunological responses[14,45–48]. Chronic administration of ethanol to animals leads to the generation of adduct-specific circulating immunoglobulins; such antibodies have been found in sera from patients with alcoholic hepatitis or cirrhosis[45,47]. In addition to humoral immune responses, acetaldehyde-modified epitopes on the cell surface have been shown to induce the generation of cytotoxic T-cells specific to acetaldehyde-altered cells[49]. The exact clinical significance of the immune response to acetaldehyde-modified proteins still remains unclear. Although there is some supporting evidence that alcohol-altered proteins have a role in mediating alcoholic liver injury, it is not explicitly known whether the immune response to such proteins represents a cause or a consequence of alcoholic liver disease[50]. Although covalent modifications due to acetaldehyde treatment only mildly affected the kinetic properties of cytosolic CAs, it is conceivable that autoantibodies, whenever present, could have more deleterious effects on the catalytic function of CAs *in vivo*. We also confirmed that the serum autoantibodies almost equally can recognize the modified CA II as compared with the native enzyme. In the immunoassay, the mean absorbance values for the acetaldehyde-modified (100 mM) CA II were 95.50% of the control values (native CA II) when tested in six subjects (data not shown). It is notable that the present immunoassay method detected only IgG class autoantibodies against the CA isozymes. Since the previous studies have indicated

significant induction in IgA autoantibody responses, the development of suitable immunoassays for IgA class antibodies will be an important goal of future studies.

Conclusions

Acetaldehyde adduct formation with different proteins is considered an important mechanism for induction of alcohol-induced organ damage. Here, we investigated acetaldehyde adduct formation *in vitro* with recombinant human cytosolic CA enzymes. High-resolution mass spectrometric analysis indicated that acetaldehyde most efficiently formed covalent adducts with CA II[40] and CA XIII (shown in the present study). In reducing conditions, CA XIII formed more adducts (up to 25) than it contains lysine residues ($n = 16$) in its primary structure. Although lysine residues are considered the preferred target amino acids for acetaldehyde binding, the results suggested that other amino acids, such as arginine and cysteine, are involved. Acetaldehyde binding only slightly decreased CA catalytic activity levels as determined by stopped-flow CA activity assay. Acetaldehyde-derived modifications on protein surfaces can induce autoantibody responses, which have been described in alcoholic patients. However, no IgG class autoantibodies against CA I, II, or VII were observed in the sera of alcohol abusers.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the article.

References

1. Freeman TL, Tuma DJ, Thiele GM, Klassen LW, Worrall S, Niemelä O et al. Recent advances in alcohol-induced adduct formation. Alcohol Clin Exp Res 2005;29:1310–1316.
2. Niemelä O, Parkkila S, Ylä-Herttuala S, Halsted C, Witztum JL, Lanca A et al. Covalent protein adducts in the liver as a result of ethanol metabolism and lipid peroxidation. Lab Invest 1994;70:537–546.
3. Lieber CS. Medical disorders of alcoholism. N Engl J Med 1995;333:1058–1065.
4. Niemelä O, Parkkila S, Koll M, Preedy VR. Generation of protein adducts with malondialdehyde and acetaldehyde in muscles with predominantly type I or type II fibers in rats exposed to ethanol and the acetaldehyde dehydrogenase inhibitor cyanamide. Am J Clin Nutr 2002;76:668–674.
5. Tuma DJ, Klassen LW. Immune responses to acetaldehyde–protein adducts: role in alcoholic liver disease. Gastroenterology 1992;103:1969–1973.
6. Niemelä O. Aldehyde–protein adducts in the liver as a result of ethanol-induced oxidative stress. Front Biosci 1999;4:D506–D513.
7. Sorrell MF, Tuma DJ. The functional implications of acetaldehyde binding to cell constituents. Ann N Y Acad Sci 1987;492:50–62.
8. Nicholls R, de Jersey J, Worrall S, Wilce P. Modification of proteins and other biological molecules by acetaldehyde: adduct structure and functional significance. Int J Biochem 1992;24:1899–1906.
9. Worrall S, de Jersey J, Nicholls R, Wilce P. Acetaldehyde/protein interactions: are they involved in the pathogenesis of alcoholic liver disease? Dig Dis 1993;11:265–277.
10. Niemelä O, Parkkila S. Alcoholic macrocytosis—is there a role for acetaldehyde and adducts? Addict Biol 2004;9:3–10.
11. Fowles LF, Beck E, Worrall S, Shanley BC, de Jersey J. The formation and stability of imidazolidinone adducts from acetaldehyde and model peptides. A kinetic study with implications for protein modification in alcohol abuse. Biochem Pharmacol 1996;51:1259–1267.
12. Niemelä O. Distribution of ethanol-induced protein adducts *in vivo*: relationship to tissue injury. Free Radic Biol Med 2001;31:1533–1538.
13. Niemelä O. Acetaldehyde adducts of proteins: diagnostic and pathogenic implications in diseases caused by excessive alcohol consumption. Scand J Clin Lab Invest Suppl 1993;213:45–54.
14. Lieber CS. Metabolic effects of acetaldehyde. Biochem Soc Trans 1988;16:241–247.
15. Setshedi M, Wands JR, Monte SM. Acetaldehyde adducts in alcoholic liver disease. Oxid Med Cell Longev 2010;3:178–185.
16. Tuma DJ, Hoffman T, Sorrell MF. The chemistry of acetaldehyde–protein adducts. Alcohol Alcohol Suppl 1991;1:271–276.
17. Donohue TM Jr, Tuma DJ, Sorrell MF. Acetaldehyde adducts with proteins: binding of [^{14}C]acetaldehyde to serum albumin. Arch Biochem Biophys 1983;220:239–246.
18. San George RC, Hoberman HD. Reaction of acetaldehyde with hemoglobin. j Biol Chem 1986;261:6811–6821.
19. Tuma DJ, Jennett RB, Sorrell MF. The interaction of acetaldehyde with tubulin. Ann N Y Acad Sci 1987;492:277–286.
20. Jennett RB, Sorrell MF, Johnson EL, Tuma DJ. Covalent binding of acetaldehyde to tubulin: evidence for preferential binding to the alpha-chain. Arch Biochem Biophys 1987;256:10–18.
21. Jennett RB, Saffari-Fard A, Sorrell MF, Smith SL, Tuma DJ. Increased covalent binding of acetaldehyde to calmodulin in the presence of calcium. Life Sci 1989;45:1461–1466.
22. Xu DS, Jennett RB, Smith SL, Sorrell MF, Tuma DJ. Covalent interactions of acetaldehyde with the actin/microfilament system. Alcohol Alcohol 1989;24:281–289.
23. Tuma DJ, Newman MR, Donohue TM Jr, Sorrell MF. Covalent binding of acetaldehyde to proteins: participation of lysine residues. Alcohol Clin Exp Res 1987;11:579–584.
24. Jukkola A, Niemelä O. Covalent binding of acetaldehyde to type III collagen. Biochem Biophys Res Commun 1989;159:163–169.
25. Gaines KC, Salhany JM, Tuma DJ, Sorrell MF. Reaction of acetaldehyde with human erythrocyte membrane proteins. FEBS Lett 1977;75:115–119.
26. Kawahara H, Matsuda Y, Tsuchishima M, Wang XE, Takada A. Effects of ethanol and acetaldehyde on the maturation of hepatic secretory glycoproteins. Alcohol Alcohol Suppl 1993;1A:29–35.
27. Bootorabi F, Jänis J, Smith E, Waheed A, Kukkurainen S, Hytönen V et al. Analysis of a shortened form of human carbonic anhydrase VII expressed *in vitro* compared to the full-length enzyme. Biochimie 2010;92:1072–1080.
28. Parkkila S. An overview of the distribution and function of carbonic anhydrase in mammals. Exs 2000;79–93.
29. Tashian RE. Genetics of the mammalian carbonic anhydrases. Adv Genet 1992;30:321–356.
30. Carter N, Wistrand PJ, Lönnerholm G. Carbonic anhydrase localization to perivenous hepatocytes. Acta Physiol Scand 1989;135:163–167.
31. Parkkila S, Parkkila AK, Juvonen T, Rajaniemi H. Distribution of the carbonic anhydrase isoenzymes I, II, and VI in the human alimentary tract. Gut 1994;35:646–650.

32. Kumpulainen T, Korhonen LK. Immunohistochemical localization of carbonic anhydrase isoenzyme C in the central and peripheral nervous system of the mouse. J Histochem Cytochem 1982;30:283–292.
33. Cammer W, Downing M. Localization of the multifunctional protein CAD in astrocytes of rodent brain. J Histochem Cytochem 1991;39:695–700.
34. Lakkis MM, O'Shea KS, Tashian RE. Differential expression of the carbonic anhydrase genes for CA VII (Car7) and CA-RP VIII (Car8) in mouse brain. J Histochem Cytochem 1997;45:657–662.
35. Lehtonen J, Shen B, Vihinen M, Casini A, Scozzafava A, Supuran CT et al. Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family. J Biol Chem 2004;279:2719–2727.
36. Hu PY, Waheed A, Sly WS. Partial rescue of human carbonic anhydrase II frameshift mutation by ribosomal frameshift. Proc Natl Acad Sci USA 1995;92:2136–2140.
37. Parkkila S, Kaunisto K, Rajaniemi L, Kumpulainen T, Jokinen K, Rajaniemi H. Immunohistochemical localization of carbonic anhydrase isoenzymes VI, II, and I in human parotid and submandibular glands. J Histochem Cytochem 1990;38:941–947.
38. Hilvo M, Innocenti A, Monti SM, De Simone G, Supuran CT, Parkkila S. Recent advances in research on the most novel carbonic anhydrases, CA XIII and XV. Curr Pharm Des 2008;14:672–678.
39. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.
40. Bootorabi F, Jänis J, Valjakka J, Isoniemi S, Vainiotalo P, Vullo D et al. Modification of carbonic anhydrase II with acetaldehyde, the first metabolite of ethanol, leads to decreased enzyme activity. BMC Biochem 2008;9:32.
41. Khalifah RG. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. J Biol Chem 1971;246:2561–2573.
42. Niemelä O, Juvonen T, Parkkila S. Immunohistochemical demonstration of acetaldehyde-modified epitopes in human liver after alcohol consumption. J Clin Invest 1991;87:1367–1374.
43. Metz B, Kersten GF, Hoogerhout P, Brugghe HF, Timmermans HA, de Jong A et al. Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. J Biol Chem 2004;279:6235–6243.
44. McMurtrie HL, Cleary HJ, Alvarez BV, Loiselle FB, Sterling D, Morgan PE et al. The bicarbonate transport metabolon. J Enzyme Inhib Med Chem 2004;19:231–236.
45. Niemelä O, Klajner F, Orrego H, Vidins E, Blendis L, Israel Y. Antibodies against acetaldehyde-modified protein epitopes in human alcoholics. Hepatology 1987;7:1210–1214.
46. Israel Y, Hurwitz E, Niemelä O, Arnon R. Monoclonal and polyclonal antibodies against acetaldehyde-containing epitopes in acetaldehyde–protein adducts. Proc Natl Acad Sci USA 1986;83:7923–7927.
47. Worrall S, de Jersey J, Shanley BC, Wilce PA. Antibodies against acetaldehyde-modified epitopes: an elevated IgA response in alcoholics. Eur J Clin Invest 1991;21:90–95.
48. Worrall S, De Jersey J, Shanley BC, Wilce PA. Acetaldehyde-modified proteins and associated antibodies in ethanol-fed rats. Alcohol Alcohol Suppl 1991;1:255–260.
49. Terabayashi H, Kolber MA. The generation of cytotoxic T lymphocytes against acetaldehyde-modified syngeneic cells. Alcohol Clin Exp Res 1990;14:893–899.
50. Klassen LW, Tuma D, Sorrell MF. Immune mechanisms of alcohol-induced liver disease. Hepatology 1995;22:355–357.