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Characterization of acrolein-induced protein cross-links

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Abstract
Lipid peroxidation products contribute to protein aggregation that occurs during oxidative stress in a number of degenerative disorders. Acrolein (ACR), a highly toxic lipid peroxidation aldehyde, is a strong cross-linking agent of cellular components such as proteins. To understand the mechanisms of oxidative stress-induced protein aggregation, this study characterized the ACR modification of chain B from bovine insulin by mass spectrometry. To identify the cross-linking sites, the ACR-treated peptide was digested with a protease and the resulting peptides were analysed by liquid chromatography-tandem mass spectrometry. Inter- and intra-molecular cross-linking adducts were identified between amino groups and the side chain of histidine in the peptide. These results indicated that the ACR-induced cross-links were accompanied by two reactions, namely Michael addition and Schiff base formation. In conclusion, the use of mass spectrometric techniques provided chemical evidence for protein cross-linking with ACR.

Keywords: *Acrolein, cross-link, lipid peroxidation, mass spectrometry, protein modification*

Abbreviations: *ACR, acrolein; CBB, Coomassie brilliant blue; FDP, formyl-dehydropiperidino; MP, methylpyridinium; insulin B chain, chain B from bovine insulin; HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.*

Introduction

The oxidative modification of proteins and subsequent accumulation of these modified proteins occur in cells during ageing, oxidative stress and in various pathological states including premature diseases, muscular dystrophy, neurodegenerative disorders, rheumatoid arthritis and atherosclerosis [1]. Important agents that give rise to the modification of a protein may be represented by reactive aldehydic intermediates, such as ketoaldehydes, 2-alkenals and 4-hydroxy-2-alkenals [2,3]. These reactive aldehydes are considered important mediators of cell damage due to their ability to covalently modify biomolecules, which can disrupt important cellular functions and cause protein denaturation [2].

Acrolein (ACR), an unpleasant and troublesome byproduct of overheated organic matter, occurs as a ubiquitous pollutant in the environment, e.g. the incomplete combustion of plastic materials, cigarette smoking and overheating frying oils. ACR is formed endogenously through oxidation reactions such as

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lipid peroxidation, polyamine oxidation by amine oxidase and myeloperoxidase-catalysed amino acid oxidation [2–4]. A number of reports have appeared describing the damaging effects of ACR on tracheal ciliatory movement and the pulmonary wall [5]. It has also been shown that ACR causes chromosomal aberrations and point mutations and reduces the colony-forming efficiency of mammalian cells [6]. In addition, ACR has been implicated in neurodegenerative disorders such as Alzheimer's disease as a possible key factor in neuronal degeneration [7].

Among α,β-unsaturated aldehydes, ACR is by far the strongest electrophile and therefore shows the highest reactivity toward nucleophiles such as proteins. Nucleophilic groups present in a peptide sequence may undergo Michael reactions with the electrophilic double bond of ACR or give rise to nucleophilic additions on the carbonyl group to form Schiff bases [1]. ACR is known to form four different types of adducts, namely aldimine-, propanal-, methylpyridinium (MP)- and formyl-dehydropiperidino (FDP)-type adducts (Figure 1) [8–12].

Further reactions of these ACR adducts are also possible, including formation of cross-links with proteins and nucleic acids. Recently, Kurtz and Lloyd [13] reported the electrophoretic evidence for cross-linking between DNA and peptides by ACR, although the cross-linked products were not isolated and characterized. Carbone et al. [14] proposed the formation of an intramolecular cross-link by an ACR molecule in ACR-treated angiotensin I. Formation of protein cross-links may be important in the initial stages of protein aggregation. However, the evidence for ACR cross-links forming in proteins has been sparse.

The purpose of this study is to characterize the protein cross-links formed upon ACR treatment by mass spectrometric techniques. Thus, a model peptide, chain B from bovine insulin (insulin B chain), was exposed to ACR, and the modified peptides were digested with endoproteinase Asp-N and analysed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography-mass spectrometry (LC-MS) to characterize the ACR-induced protein cross-links.

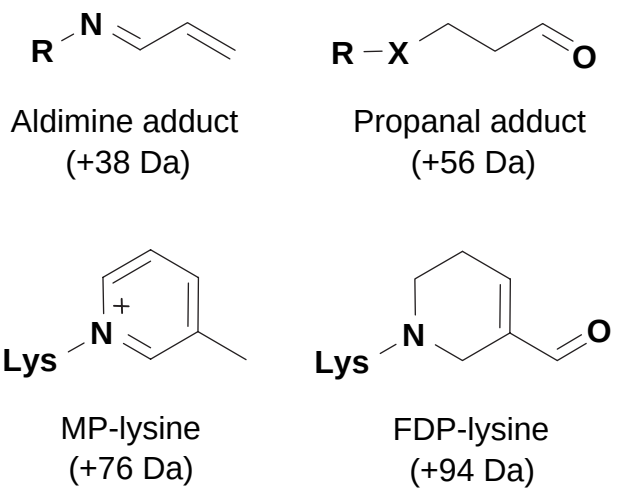


Figure 1. Chemical structures of aldimine-, propanal-, MP- and FDP-type ACR adducts. The numbers represent the increments of Mw as adducts are formed.

Materials and methods

Materials

ACR and insulin B chain were obtained from Sigma (St Louis, MO, USA). Formic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Endoproteinase Asp-N was obtained from Roche Diagnostics (Basel, Switzerland). Sodium cyanoborohydride ($NaCNBH_3$) was purchased from Sigma-Aldrich (St Louis, MO, USA).

Reaction of insulin B chain with ACR

Insulin B chain (1 mg/mL) was incubated in a 1:1 or 1:2 molar ratio with ACR in 50 mM sodium phosphate buffer (pH 7.2) at 37°C for 24 h. The reaction was terminated by centrifugal filtration (Microcon 3, molecular weight cut-off of 3000; Millipore) to remove the low molecular weight reactants. Control experiments were performed under the same conditions without addition of ACR. The stabilization of the aldimine-type adduct was performed by the addition of a freshly prepared aqueous solution of $NaCNBH_3$.

MALDI-TOF MS analysis

Native or ACR-treated insulin B chains were mixed with a 25% solution of sinapinic acid containing 80% acetonitrile and 0.1% trifluoroacetic acid. The mixture was spotted and dried on stainless steel targets at room temperature. The analyses were performed using an UltraFLEX MALDI-TOF MS (Bruker Daltonics, Ltd., Germany). All analyses were carried out in the positive ion mode and the instrument was calibrated immediately prior to each series of studies.

LC-MS analysis of native and ACR-modified insulin B chains

Native and ACR-modified insulin B chains were analysed by reversed-phase high performance liquid chromatography (HPLC) [15]. Separation of products was carried out on a nanospace SI-1 HPLC system (Shiseido, Tokyo, Japan) using a Capcell Pak C18 UG300 column (Shiseido). Samples were eluted with a linear gradient of 0.1% formic acid in water (solvent A)-acetonitrile (solvent B) (time = 0 min, 20% B; 0–5 min, 20% B; 5–25 min, 35% B; 25–35 min, 45% B). The flow rate was 0.2 mL/min and the column temperature was controlled at 40°C. The

elution profiles were monitored by absorbance at 215 nm. The MS analyses were performed on an LCQ ion trap mass system (Thermo Fisher Scientific, San Jose, CA) equipped with an electrospray ion source using a spray voltage of 5 kV and a capillary temperature of 260°C.

SDS-PAGE

The samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 16–20% gradient gels. Sample buffer and running buffer used the tricine-HCl buffer system (NOVEX-Tricine Gel System, Invitrogen). The peptide was stained with Coomassie brilliant blue (CBB).

Peptide mapping and tandem mass spectrometry (MS/MS) analysis

Native and ACR-modified insulin B chains were digested with endoproteinase Asp-N in 50 mM sodium phosphate buffer (pH 7.2) at 37°C for 24 h using an enzyme:substrate ratio of 1:50 (w/w). Peptide samples were analysed by reversed-phase HPLC, using the same system described above, with a Capcell Pak C18 UG120 column (Shiseido). Samples were eluted with a linear gradient of 0.1% formic acid in water (solvent A)-acetonitrile (solvent B) (time = 0 min, 5% B; 0–7 min, 5% B; 7–10 min, 15% B; 10–60 min, 50% B).

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed on an LCQ ion trap mass system equipped with an electrospray ion source using a spray voltage of 5 kV and a capillary temperature of 260°C. Collision induced dissociation experiments in the positive ion mode were performed by setting the relative collision energy at 25% using helium as the collision gas.

Results and discussion

Modification behaviour of ACR on insulin B chain

To characterize the mechanisms of ACR-induced protein cross-links, we used as a model peptide the insulin B chain from sequence FVNQHLC*GSH LVEALYLVC*GERGFFYTPKA (C*: Cys-SO_3H), which contains four main possible modification sites of ACR: the N-terminal amino acid residue (Phe1), two histidine residues (His5 and His10) and one lysine residue (Lys29). ACR is known to form four different types of adducts, namely aldimine-, propanal-, MP- and FDP-type adducts, resulting in increments of 38, 56, 76 and 94 Da, respectively, in the mass value of the peptide (Figure 1). We first examined the modification behaviour of ACR with the peptide during 24 h of incubation. As shown in Figure 2A, MALDI-TOF MS analysis of the native insulin B chain revealed a peak at *m/z* 3496, which was in agreement with the theoretical molecular mass derived from the sequence for insulin B chain. When insulin B chain was incubated in a 1:1 or 1:2 molar

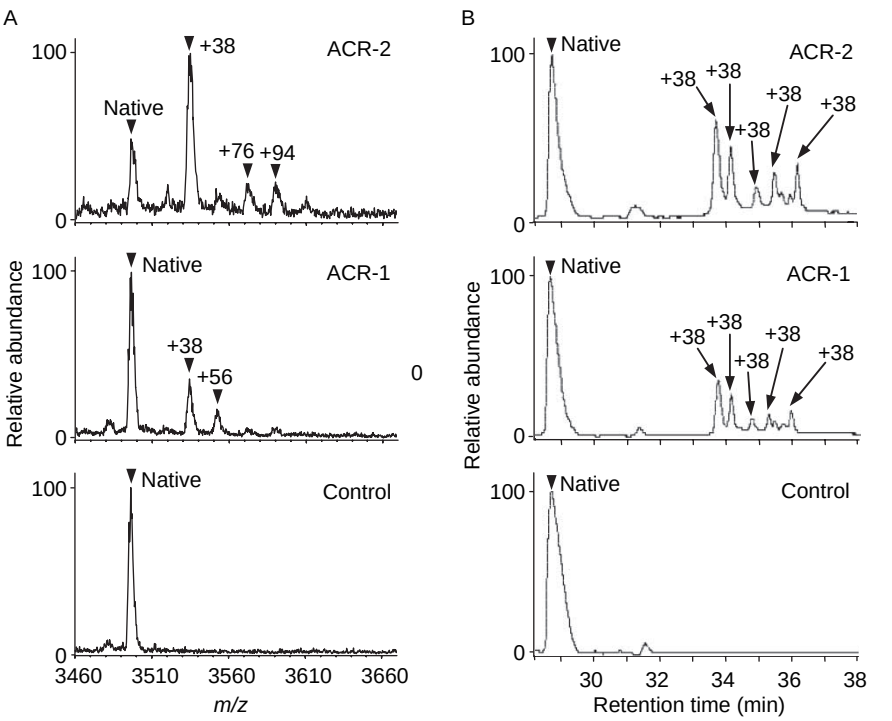


Figure 2. ACR modification of insulin B chain. Insulin B chain (1 mg/mL) was incubated in a 1:1 (ACR-1) or 1:2 (ACR-2) molar ratio with ACR in 50 mM sodium phosphate buffer (pH 7.2) at 37°C. (A) MALDI-TOF MS spectra of native and ACR-modified insulin B chains. (B) LC-MS profiles of native and ACR-modified insulin B chains.

ratio with ACR in 50 mm sodium phosphate buffer (pH 7.2) for 24 h, some ACR-modified insulin B chain was observed (Figure 2A). On the basis of their mass values, we concluded that the peaks originated from the generation of aldimine- (+38 Da), propanal- (+56 Da), MP- (+76 Da) and FDP-type (+94 Da) adducts. The peak at *m/z* 3534, which corresponded to the increment of 38 Da in the peptide mass, was most prevalent. This result suggests that the main products were the aldimine-type adducts under these experimental conditions. Subsequently, aliquots of native and ACR-modified insulin B chains were analysed by LC-MS equipped with a UV detector. As shown in Figure 2B, several peaks were detected on the chromatogram by UV (215 nm) detection and the individual peaks were then assigned by mass spectrometry (data not shown). LC-MS analysis of the native insulin B chain gave a molecular mass of 3496 Da. The new peaks gave the same molecular mass, at *m/z* 3534 (+38 Da), which corresponded to the generation of the aldimine-type adducts. The corresponding mass shift indicates that the main products are aldimine-type adducts in the peptide, including at least five different structures.

Detection of ACR-induced protein cross-links

Previous evidence for unsaturated aldehyde cross-links forming in proteins has been sparse. It is likely that these reactions occur frequently, but the resulting aldimine-type adducts are difficult to detect due to their hydrolytic instability [16]. ACR-aldimine adducts dissociate reversibly to the component aldehyde and amine and can be detected as stable covalent species in the presence of $NaCNBH_3$. Therefore, to detect the cross-linking adducts in the model peptide, ACR-treated insulin B chain was reduced with $NaCNBH_3$ then analysed by SDS-PAGE (Figure 3). When insulin B chain was incubated in a 1:1 or 1:2 molar ratio with ACR in 50 mM sodium phosphate buffer (pH 7.2) for 24 h, native bands (3.5 kDa) were significantly decreased on the gel (lanes 5 and 6, lower arrowhead), but mobility shifts were only slightly observed (lanes 5 and 6, middle arrowhead). However, $NaCNBH_3$ reduction of the ACR-treated peptide resulted in complete mobility shifts of the native peptide (lanes 3 and 4). In comparison with the unreduced condition, the middle bands were significantly increased in the reduced condition, suggesting the formation of aldimine-type adducts such as intramolecular cross-links. In addition, higher molecular weight bands, suggesting intermolecular cross-links, also appeared (lanes 3 and 4, upper arrowhead). This result strongly suggests that ACR forms inter- or intramolecular cross-linking adducts in the peptide.

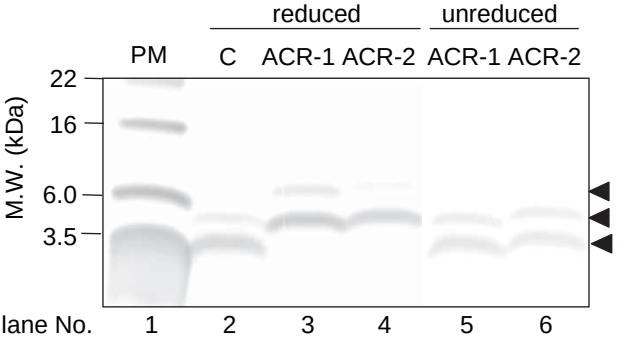


Figure 3. Formation of cross-linking adducts in the ACR-modified insulin B chain. Insulin B chain (1 mg/mL) was incubated in a 1:1 (ACR-1) or 1:2 (ACR-2) molar ratio with ACR in 50 mM sodium phosphate buffer (pH 7.2) at 37°C. The samples following reduction with $NaCNBH_3$ were separated by SDS-PAGE. The separated peptides were detected by CBB staining. Lane 1, protein marker (PM); lane 2, control insulin B chain (C); lane 3, reduced ACR-1; lane 4, reduced ACR-2; lane 5, unreduced ACR-1; lane 6, unreduced ACR-2. The upper arrow indicates intermolecular cross-links; the middle arrow indicates ACR-adducts including aldimine-type adducts, such as intramolecular cross-links; the lower arrow indicates native protein.

Detection of ACR modified fragments in protease digested insulin B chain by HPLC analysis

To identify the cross-linking sites in the model peptide, ACR-treated insulin B chain (ACR:insulin B chain = 1:1) following the reduction with $NaCNBH_3$ was digested with endoproteinase Asp-N and the resulting peptides were subjected to LC-MS. The protease digestion of insulin B chain theoretically generates three fragments, namely FVNQHL (F1), C^*GSHLVEALYLV (F2, C^*: Cys-SO_3H) and C^*GERGFFYTPKA (F3, C^*: Cys-SO_3H). As shown in Figure 4A, limited proteolysis of native insulin B chain indeed gave three peaks, a, b and c. Peaks a, b and c were identified to be F1, F3 and F2, respectively. The other peaks (such as the peak at RT = 28.5 min) were not identified as fragments of the insulin B chain. The ACR-modified insulin B chain digested with the protease was also analysed by LC-MS. After modification by ACR, peaks a (F1) and c (F3) decreased significantly and new peaks (d–g) appeared (Figure 4B). In the reduced condition, peaks d–g disappeared and new peaks (h–l) appeared (Figure 4C), strongly suggesting that they contain aldimine-type adducts.

Characterization of cross-linking sites in ACR-modified insulin B chain by MS/MS analysis

Mass spectrometric analysis of digests of cross-linked proteins is known to be a powerful tool to identify cross-linking sites [17]. However, the identification of cross-linked sites in proteins with a Schiff base structure is unprecedented. To characterize the cross-linking sites of the ACR-modified insulin B chain, both unmodified fragments (a, b and c) and

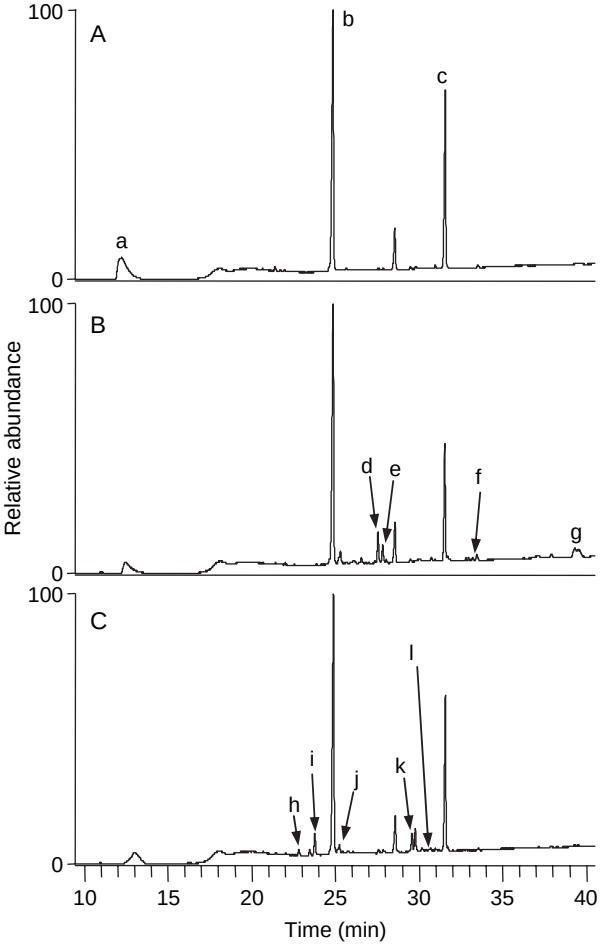


Figure 4. HPLC profiles (abs. 215 nm) of digested native and ACR-modified insulin B chains. Native and ACR-treated insulin B chain (ACR:insulin B chain = 1:1) following the reduction with $NaCNBH_3$ was digested with endoproteinase Asp-N and the resulting peptides were subjected to LC-MS. (A) Chromatograms of digested native insulin B chain. (B) Chromatograms of digested ACR-modified insulin B chain. (C) Chromatograms of digested ACR-modified insulin B chain following reduction with $NaCNBH_3$. Molecular masses and modified structures of each peak (a–l) are shown in Table I.

ACR-modified fragments (d–l) were analysed by LC-MS/MS. Four of these candidate cross-links have been confirmed by tandem mass spectrometric analyses of the corresponding cross-linked peptides. MS/MS analysis of peak h (F1 + 40 Da) revealed the singly charged C-terminal product ions (y4 and y5) and H_2O-loss fragment (y3-18) (Figure 5A). Furthermore, there was a 40 Da increase compared to the native peptide fragment ions (a2, a5, b2, b3, b4, b5 and c5) and H_2O-loss fragment (a5-18). These results confirmed that the aldimine-type adduct is associated with the N-terminus of Phe1. Figure 5B shows the MS/MS spectrum of peak i (F1 + 40 Da), which has the same $[M+H]^+$ as peak h. The fragment ions (a5, b5 and c5) and H_2O loss fragment (a5-18) showed a 40 Da increase compared to those in the native peptide and peak h. On the other hand, new fragments (b2 + His, a1 + y2, b1 + y2, a2 + y2 and b2 + y2) associated with the ACR-bridge were observed. These data indicated that intermolecular cross-linking occurs between Phe1

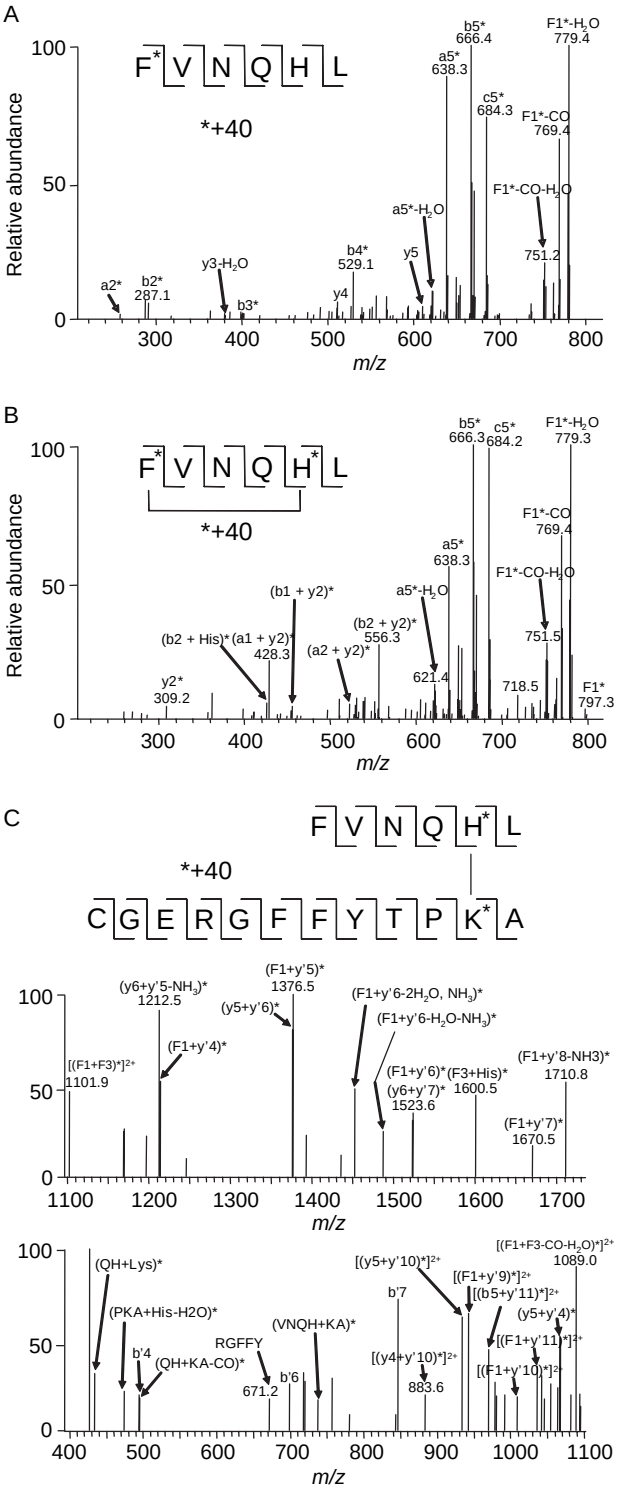


Figure 5. LC-MS/MS spectra of the peptides from the ACR-modified insulin B chain following reduction with $NaCNBH_3$. (A) MS/MS spectrum of the $[M+H]^+$ ion at *m/z* 797.4 from peak h (F1 + 40 Da). Label* denotes modification by ACR. (B) MS/MS spectrum of the $[M+H]^+$ ion at *m/z* 797.4 from peak i (F1 + 40 Da). Label* denotes cross-linking by ACR. (C) MS/MS spectrum of the $[M+2H]^{2+}$ ion at *m/z* 1110.5 from peak j (F1 + F3 + 40 Da). Label* denotes cross-linking by ACR.

and His5. Figure 5C shows the MS/MS spectrum of peak j (F1 + F3 + 40 Da). The MS/MS spectrum revealed F3-related product ions (b'4, b'6 and b'7). Fragments associated with the ACR-bridge were also observed. Characteristically, all of the fragments included both His5 from F1 and Lys29 from F3 such as (PKA + His) and (QH + Lys). This indicates that cross-linking occurs between His5 and Lys29. Table I summarizes the results of the MS analysis of the endoproteinase Asp-N digested ACR-modified insulin B chain. From MS analysis, we identified the formation of cross-linking adducts in both amino groups [N-terminus (Phe1) or Lys29] and the histidine residues (His5 or His10).

Proposed mechanism of protein cross-linking by ACR

ACR can display different reactivities toward nucleophilic groups present in a peptide sequence. These nucleophilic groups can either react with the electrophilic double bond of ACR or give rise to nucleophilic addition on the carbonyl group to form a Schiff base (aldimine-type adduct) [3]. ACR can also react via Michael addition with the nitrogens of the imidazole rings of histidine, the thiol groups of cysteine or the amino groups of the N-terminus and lysine. Propanal- and FDP-type adducts, which contain carbonyl structures, are also known to form with ACR [3,8-11]. Incubation of insulin B chain with ACR resulted in a dose-dependent increase in protein carbonyl formation, suggesting that ACR modifies the insulin B chain by Michael reaction (data not shown). However, MS analysis did not show the remarkable formation of this protein carbonyl. A protein carbonyl group can react with a protein amino group to form a Schiff base. Previous reports indicate that aldehyde-induced carbonyl structures are important for the formation of protein cross-linking [18,19]. In addition, hydralazine targets carbonyl-containing ACR adducts, forming hydrazones that may prevent participation by modified proteins in nucleophilic additions that generate inter- and intramolecular cross-links [20]. These results and observations suggest that inter- and intramolecular cross-linking adducts form between amino groups and the side chain of histidine in this peptide (Scheme 1).

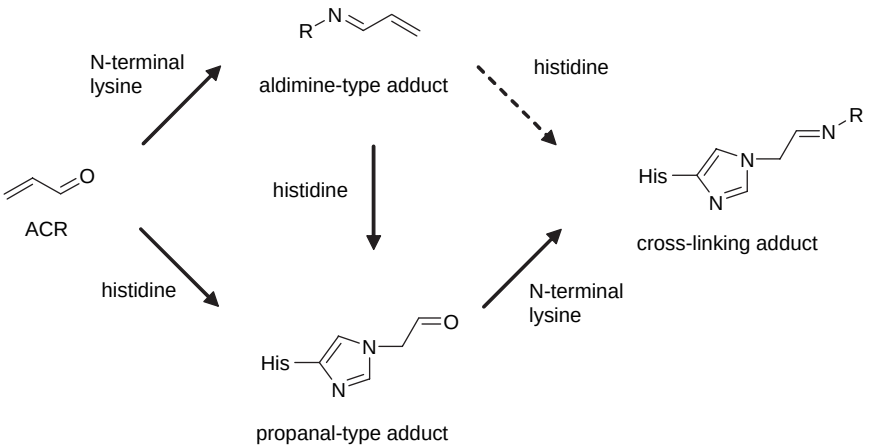
Insulin B chain contains two key sites (N-terminus Phe1 or Lys29) for cross-link formation by ACR, making the formation of oligomers a definite possibility. Oligomers were not detected in this study; however, a change in the protein profile with different concentrations of ACR was observed by MALDI-TOF MS (Figure 2A). With higher concentrations of ACR (ACR-2), formation of MP-type adducts is observed. Previously, we investigated the reaction of proteins with ACR and identified a novel ACR-lysine adduct, MP-lysine [12]. The formation of MP-lysine may be reasonably explained by a mechanism involving the formation of a Schiff base derivative as the first intermediate. The Schiff base further reacts with a second acrolein molecule via Michael addition to generate an imine derivative. The subsequent conversion of this imine derivative to the final product (MP-lysine) obviously requires two oxidation steps and intramolecular cyclization, but its detailed mechanism is not yet clear. Although MP-lysine was detected as a minor product in the reaction of ACR with the lysine derivative (Figure 4), these pyridinium adducts are highly stable end products. Therefore, the formation of MP-type adducts may disrupt the protein cross-links in this study, preventing the formation of higher oligomers.

A number of reports have appeared describing the damaging effects of ACR on proteins and DNA modifications [21–23]. Such modifications ensure that ACR disrupts a wide range of cellular and molecular processes in exposed tissues, including alterations in the activity of various transcription factors, enzyme activities, mitochondrial processes and redox regulatory pathways [24,25]. Consistent with endogenous ACR production via lipid

Table I. Summary of the Asp-N digested peptides from ACR-modified insulin B chain.

Peak	Observed mass	* Δm (Da)	Proposed structure	Proposed modification site
a	757.4	—	F3	—
b	1423.7	—	F1	—
c	1351.6	—	F2	—
d	795.5	38	F1 + aldimine-type	F1
e	795.5	38	F1 + aldimine-type	F1
f	795.5	38	F1 + aldimine-type	F1
g	1389.6	38	F2 + aldimine-type	N-terminus (Cys7)
h	797.4	40	F1 + aldimine-type (reduced)	N-terminus (Phe1)
i	797.4	40	F1 + aldimine-type (reduced)	N-terminus (Phe1) + His5 (cross-link)
j	1110.5^{2+}	40	F1 + F3 + aldimine-type (reduced)	His5 + Lys29 (cross-link)
k	1074.9^{2+}	40	F1 + F2 + aldimine-type (reduced)	N-terminus (Phe1) + His10 (cross-link)
l	1408.1^{2+}	40	F2 + F3 + aldimine-type (reduced)	His10 + Lys29 (cross-link)

*Δm (Da) is the increase in weight against native insulin B chain.



Scheme 1. Proposed mechanism of protein cross-linking by ACR.

peroxidation cascades, ACR-adducted proteins have been detected in the affected tissues of a number of degenerative conditions known to involve oxidative stress, including Alzheimer's disease and atherosclerosis [26,27]. The level of ACR is increased in the amygdala and hippocampal/parahippocampal gyrus of Alzheimer's disease patients compared to normal controls [28]. Furthermore, ACR induced the formation of Tau-aggregates *in vitro* [29]. The reaction of ACR with nucleophilic amino acids of Tau results in the formation of Michael adducts [30]. Subsequent inter- and intramolecular reactions of the ACR adducts were described, yielding lysine adducts [11,20]. However, the exact structure of an ACR-based protein cross-link has not been published to date. We have provided in the present study a detailed overview of possible cross-linking structures caused by ACR. Furthermore, our results indicate that the ACR induced cross-links were accompanied by two types of reactions, namely Michael addition and Schiff base formation. These findings may contribute to the understanding of the damaging effects of lipid derived α,β-unsaturated aldehydes such as ACR in various pathological states.

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