Cross-Linking Methionine and Amine Residues with Reactive Halogen Species

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Original Contribution

Cross-linking methionine and amine residues with reactive halogen species

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ABSTRACT

Irreversible cross-links are increasingly being recognized as important posttranslational oxidative protein modifications that contribute to tissue injury during oxidative stress and inflammation. They also have a structural function in extracellular matrix proteins such as collagen IV. Likely contenders for forming such cross-links are the reactive halogen species that are generated by neutrophils and eosinophils, including hypochlorous acid, hypobromous acid, and their related haloamines. Methionine residues are kinetically preferred targets for these oxidants and oxidation can potentially result in sulfilimine ($>S{=}N-$) bonds with amines. Therefore, we investigated whether oxidation of methionine in the model peptide formyl-Met-Leu-Phe-Lys (fMLFK) produces cross-links with lysine residues, using mass spectrometry to characterize the products. As expected, the sulfoxide was the major product with each reactive halogen species. However, intra- and intermolecular cross-linked products were also formed. Isomers of an intramolecular sulfilimine were readily produced by hypobromous acid and bromamines, with hypochlorous acid forming lesser amounts. The predominant cross-link with chloramines was an intermolecular bond between the sulfur of fMLFK and the amine derived from the chloramine. Reactive halogen species also formed these sulfilimine cross-links in other peptides that contain methionine. We propose that protein cross-links involving methionine and amine residues will form via this mechanism when granulocytes are activated at sites of inflammation. Our results also support the proposal that reactive halogen species generated by the peroxidase peroxidasin could be responsible for the sulfilimine bonds that are integral to the structure of collagen IV.

Phagocytic cells contain heme peroxidases that catalyze the formation of hypohalous acids from hydrogen peroxide and a halide. These highly reactive oxidants play a central role in host defense but also contribute to inflammatory tissue damage [1,2]. Myeloperoxidase is an abundant protein in neutrophils and is also present in monocytes. It catalyzes the oxidation of chloride (Cl^-), bromide (Br^-), and the pseudo-halide thiocyanate (SCN^-) to hypochlorous acid (HOCl), hypobromous acid (HOBr), or hypothiocyanite (^-OSCN), respectively [3–6]. Eosinophil peroxidase generates ^-OSCN and HOBr but not HOCl [7–9]. It is becoming apparent that another mammalian heme peroxidase, peroxidasin 1 (also known as vascular peroxidase 1), is capable of oxidizing chloride and bromide [10,11].

HOCl and HOBr react rapidly with cysteine and methionine residues on proteins to give predominantly disulfides and methionine sulfoxide, respectively [12–16]. Chloramines and bromamines, formed by the reaction of these oxidants with amine residues, are less reactive but show a similar preference [12,17,18]. There is increasing evidence that HOCl and HOBr can promote cross-linking of biological molecules via non-disulfide bonds. For example, numerous investigators have reported that reactive halogen species promote the nonreducible cross-linking of proteins including fibronectin [19], lysozyme [20], calprotectin [21,22], and those in erythrocyte membranes [23]. Such cross-links also occur between DNA and protein [24]. Oxidation of methionine residues by HOCl was implicated in the facile oligomerization of myoglobin [25]. Although it is generally believed that oxidation of methionine by hypohalous acids yields methionine sulfoxide [26,27], it was recently demonstrated that dehydromethionine and azasulfonium salts from *N*-terminal methionine residues are major products in these reactions [28,29]. These species contain a five-membered ring joined by an $>N{-}S^+<$ bond. A related

Abbreviations:: GYGGFM, Gly-Tyr-Gly-Gly-Phe-Met; GYGGFM(S=O), Gly-Tyr-Gly-Gly-Phe-Met sulfoxide; fMLFK, formyl-Met-Leu-Phe-Lys; fM(=O)LFK, formyl-Met-Leu-Phe-Lys sulfoxide; f**M**LF**K**, intramolecular adduct of formyl-Met-Leu-Phe-Lys; *NAc*-K-fMLFK, intermolecular adduct between N_α-acetyl-lysine and formyl-Met-Leu-Phe-Lys; LC–MS, liquid chromatography–mass spectrometry; EMS, enhanced mass spectrum; EPI, enhanced product ion; SRM, selected reaction monitoring

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sulfilimine cross-link (–N═S<) between hydroxylysine-211 and methionine-93 of adjoining protomers in collagen IV has also been identified [30]. The collagen cross-links have been shown to be catalyzed by peroxidasin 1 and a reaction involving hypohalous acids has been proposed. However, the species generated *in vivo* has yet to be clearly defined [10]. These novel bonds are vital for correct folding of collagen IV. They also mask the collagen IV autoantigen in human Goodpasture disease, and failure to form them contributes to the pathogenesis of this autoimmune disease [31]. Recent data indicate that this sulfilimine cross-link arose 500 Mya and is evolutionarily conserved throughout Metazoa [32]. In view of the importance of the Met-Lys in extracellular matrix formation and autoimmunity, it is important to determine whether it can be formed by hypohalous acids and to understand the mechanism of the process.

We propose that cross-links in and between proteins, formed via the covalent attachment of methionine and amine residues, are important products of the reactions of hypohalous acids generated by mammalian peroxidases. The aim of this study was to demonstrate that reactive halogen species promote either intramolecular cross-links in peptides containing both methionine and lysine residues or intermolecular cross-links between peptides when one has a methionine residue and the other a free amine group.

Materials and methods

Materials

L-Methionine, N_α-acetyllysine, glycine, and taurine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gly-Tyr-Gly-Gly-Phe-Met (Gly-Met-enkephalin, GYGGFM)[1] and formyl-Met-Leu-Phe-Lys (fMLFK) were from Bachem (Bubendorf, Switzerland). All the reagents for buffers were of analytical grade. Hypochlorous acid solution was prepared daily by diluting the concentrated stock solution and calculating its concentration using an ε_{292} of 350 M^{-1} cm^{-1} at pH 12 [33]. HOBr solution was generated by mixing Br^- (10 mM) with OCl^- (2 mM) at pH 9 [19,33]. Hypobromous acid formation was followed spectrophotometrically

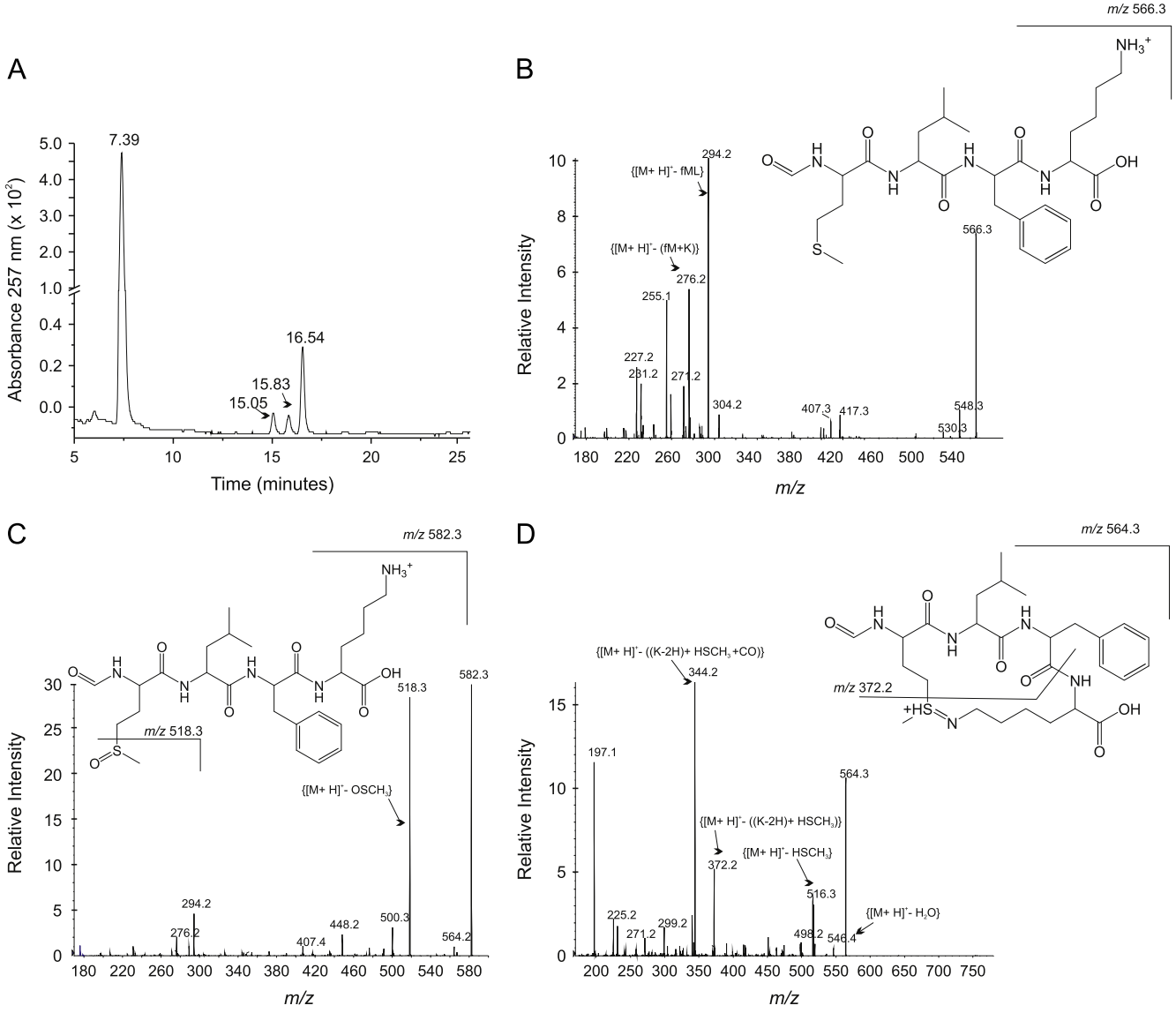

Fig. 1. Analysis of products formed in the reaction between HOBr and fMLFK. HOBr (200 μM) was added with vigorous vortexing to fMLFK (200 μM) in 20 mM phosphate buffer, pH 7.4, containing 100 μM DTPA. (A) UV chromatogram at 257 nm of the products and reactants. (B) MS/MS fragmentation pattern for the parent fMLFK (retention time of 16.54 min in (A)). (C) Proposed structure and MS/MS fragmentation pattern for the sulfoxide (fM(═O)LFK, retention time of 7.39 min in (A)). (D) Proposed structure and MS/MS fragmentation pattern for the intramolecular sulfilimines (f**M**LF**K**, retention times of 15.05 and 15.83 min in (A)).

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(ε_{329}=332 M^{-1} cm^{-1}). The HOBr was generated within 30 min of use because it is unstable [34].

Reactions of HOCl and HOBr with fMLFK

HOCl or HOBr (50–400 μM) was added with vigorous vortexing to fMLFK (200 μM) in 20 mM phosphate buffer, pH 7.4, containing 100 μM diethylenetriaminepentaacetic acid (DTPA). The reactions were stopped after 1 min by adding methionine (1 mM). DTPA was added to chelate metals and had no effect on the cross-linking reactions (data not shown).

Reactions of chloramines and bromamines with peptides

Solutions containing haloamines of N_α-acetyllysine, glycine, taurine, or ammonia were prepared by adding HOCl or HOBr to the amines, under vigorous vortexing, in 20 mM phosphate buffer, pH 7.4, containing 100 μM DTPA. Unless stated otherwise, the proportion of amine:hypohalous acid was kept at 5:1, and the hypohalous acid was added to the solution containing the amine, to avoid dihaloamine formation [35]. Immediately after forming the haloamine, this solution (200 μM haloamine) was added with vigorous vortexing to fMLFK (200 μM) in the same buffer. The reactions were stopped after 1 min, by adding an excess of methionine (1 mM). For concentration-dependence studies, 50, 100, 200, or 400 μM N_α-acetyllysine haloamine solution was added to 200 μM fMLFK. For some experiments, the final concentration of N_α-acetyllysine was increased from 1 to 5 mM. For pH-dependent and stability studies, acetate (pH 4), citrate phosphate (pH 5.5), phosphate (pH 7.4), and carbonate (pH 9) were used as buffers.

Liquid chromatography–electrospray ionization–mass spectrometry

HPLC analyses were performed with a binary pump (1200 series, Agilent, Waldbronn, Germany). For the separation, a 150 × 2.1-mm (particle size 5 μm) Luna C-18 column (Phenomenex) was used. Separation was obtained using a gradient elution of ammonium acetate (pH 6.8, 20 mM, solvent A) and acetonitrile (solvent B). The gradient started with 17% B reaching 25% in 18 min, increasing to 80% B in 1 min. The column was washed with this solvent proportion for 2 min and returned to starting conditions in 1 min, followed by reequilibration for 10 min. The flow rate was 200 μl/min. The column was maintained at 25 °C and the autoinjector at 4 °C. For the MS/MS analysis, a 4000 Q Trap mass spectrometer with an electrospray source (Applied Biosystems, Foster City, CA, USA) was used. The samples were analyzed in the positive-ion mode using enhanced mass spectrum (EMS), enhanced product ion (EPI), and selected reaction monitoring (SRM) scan modes. An SRM survey scan was performed with the following transitions: 566 to 548 and 566 to 294 *m/z* for fMLFK, 582 to 518 *m/z* for sulfoxide, 751 to 518 *m/z* for intermolecular adduct, and 564 to 372 and 564 to 344 *m/z* for intramolecular adducts. Selected reaction monitoring transitions were chosen based on EMS and EPI spectra. Each transition was obtained with a dwell time of 200 ms. The SRM analyses were performed using collision energy and collision excitation potential at 40 and 10 eV, respectively. The collision-activated dissociation gas flow was set as medium. EMS and EPI survey scans were acquired with a scan rate of 1000 amu/s and by summing two scans. The source parameters were set as follows: curtain gas, 10 psi; ion source, 4000 V; temperature, 600 °C; gas 1, 40 psi; gas 2, 50 psi; declustering potential, 60 V.

The absolute concentrations of products were determined by relating their absorbance at 257 nm due to the presence of phenylalanine to that obtained for known concentrations of the parent peptide.

Results

Reaction of fMLFK with hypohalous acids

Initially we investigated whether HOCl and HOBr could promote an intramolecular cross-link between the methionine and lysine residues in the peptide fMLFK. When analyzed by LC–MS, the parent peptide had a retention time of 16.5 min and its protonated molecule ($[M+H]^+$) had an *m/z* value of 566.3 (Figs. 1A and B). The identifiable fragment ions of fMLFK involved losses of H_2O (*m/z* 548.3) and the y_2 ion (*m/z* 294.2) corresponding to the fML moiety of the peptide (Fig. 1B). Oxidation of fMLFK by either HOCl or HOBr gave fMLFK sulfoxide (fM(=O)LFK) as the major product. It eluted at approximately 7.4 min (Fig. 1A). Its identity was confirmed by the increase in *m/z* of 16 due to the incorporation of oxygen, as well as the characteristic loss of 64 mass units for a sulfoxide [36,37] (Fig. 1C).

HOBr caused the formation of two additional products with retention times of 15.1 and 15.8 min (Fig. 1A). These products were also formed by HOCl but in much lower yields (see Fig. 2). Both products had *m/z* values of 564.3, which is 2 mass units less than the parent peptide, consistent with the formation of an

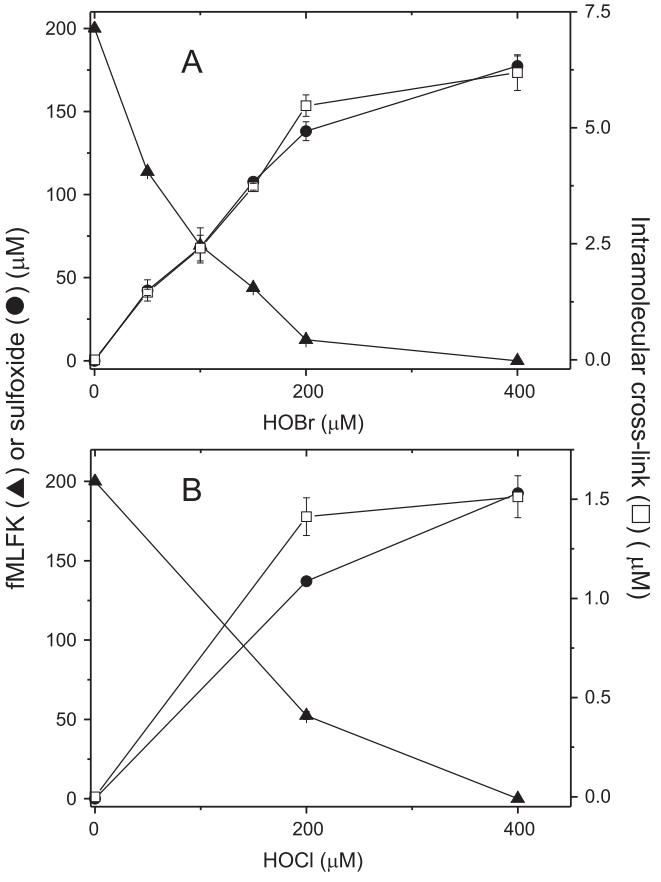

Fig. 2. The effect of the concentration of hypohalous acids on the oxidation of fMLFK. (A) HOBr or (B) HOCl was added with vigorous mixing to 200 μM fMLFK in 20 mM phosphate buffer, pH 7.4, containing 100 μM DTPA. The left *y* axis shows the concentration (μM, closed symbols) of fMLFK and formation of the sulfoxide, and the right *y* axis shows the formation of the intramolecular cross-link (μM, open symbols). Absolute concentrations of the peptide and its products were determined by relating their absorbance at 257 nm, due to the presence of phenylalanine, to that obtained for known concentrations of the parent peptide.

intramolecular sulfilimine bond (see Fig. 1D for the proposed structure). These two products had the same fragmentation pattern, indicating that they are isomers (data not shown). They also had a fragment ion with *m/z* 516.3, compatible with the loss of the $HSCH_3$ group. This is consistent with a modification involving the sulfur atom because it was not produced upon fragmentation of the parent peptide. The related fragment ion (*m/z* 518.3) was present in the fMLFK sulfoxide (loss of H_3CSO; see Fig. 1C). The two isomers also showed elimination of H_2O (*m/z* 546.4) and H_2O plus $HSCH_3$ (*m/z* 498.2) upon fragmentation (Fig. 1D). Additional support for the proposed structure was obtained with the fragment ion at *m/z* 372.2. This ion results from the elimination of the lysine residue along with the $HSCH_3$ from the amino-terminal methionine. The *m/z* ratio of 2 units less than the parent fMLFK, along with MS/MS characterization, provides strong evidence that these products contain an intramolecular cross-link between the sulfur atom of the N-terminal methionine and the side-chain amino group of lysine (f**M**LF**K**).

There was a progressive loss of fMLFK and formation of the sulfoxide with increasing concentration of HOBr (Fig. 2A). The sulfoxide accounted for the majority of the reacted peptide. Similar data were obtained with HOCl. The formation of the intramolecular cross-linked product increased to a maximum of about 2.5% of the parent with stoichiometric addition of HOBr (Fig. 2B). Less of this species was formed with HOCl.

Reaction of fMLFK with haloamines

The peptide fMLFK was reacted with the haloamines of N_α-acetyllysine to determine whether they form intramolecular sulfilimines or intermolecular cross-links between the lysine residue of the haloamine and the methionine residue in the peptide. The sulfoxide (retention time of 7.43 in Figs. 3A and B) was the major product with the bromamine (Fig. 3A) or chloramine (Fig. 3B). In addition to sulfoxide and the unreacted fMLFK (retention time of 16.5 in Fig. 3A), three products with retention times of approximately 6.6, 15.1, and 15.8 min were also detected. These products had *m/z* values of 751.4, 564.3, and 564.3, respectively. The retention times and fragmentation patterns of the products with *m/z* 564.3 were identical to those presented in Fig. 1D and correspond to the intramolecular azasulfonium salt. Tandem mass spectrometric analysis of the product at *m/z* 751.4 is compatible with the addition of the N_α-acetyllysine to fMLFK to form an intermolecular adduct (*N*Ac-K-fMLFK; Figs. 3C and 3D). The major fragment at *m/z* 518.3 corresponds to the loss of the N_α-acetyllysine in conjunction with the $HSCH_3$ moiety (Fig. 3D).

There was a progressive loss of fMLFK upon reaction with increasing concentrations of either N_α-acetyllysine chloramine or N_α-acetyllysine bromamine and a corresponding increase in the formation of the fM(=O)LFK (Fig. 4A). The bromamine was more reactive than the chloramine in that it converted more of the peptide to the sulfoxide. It also gave rise to much higher yields of

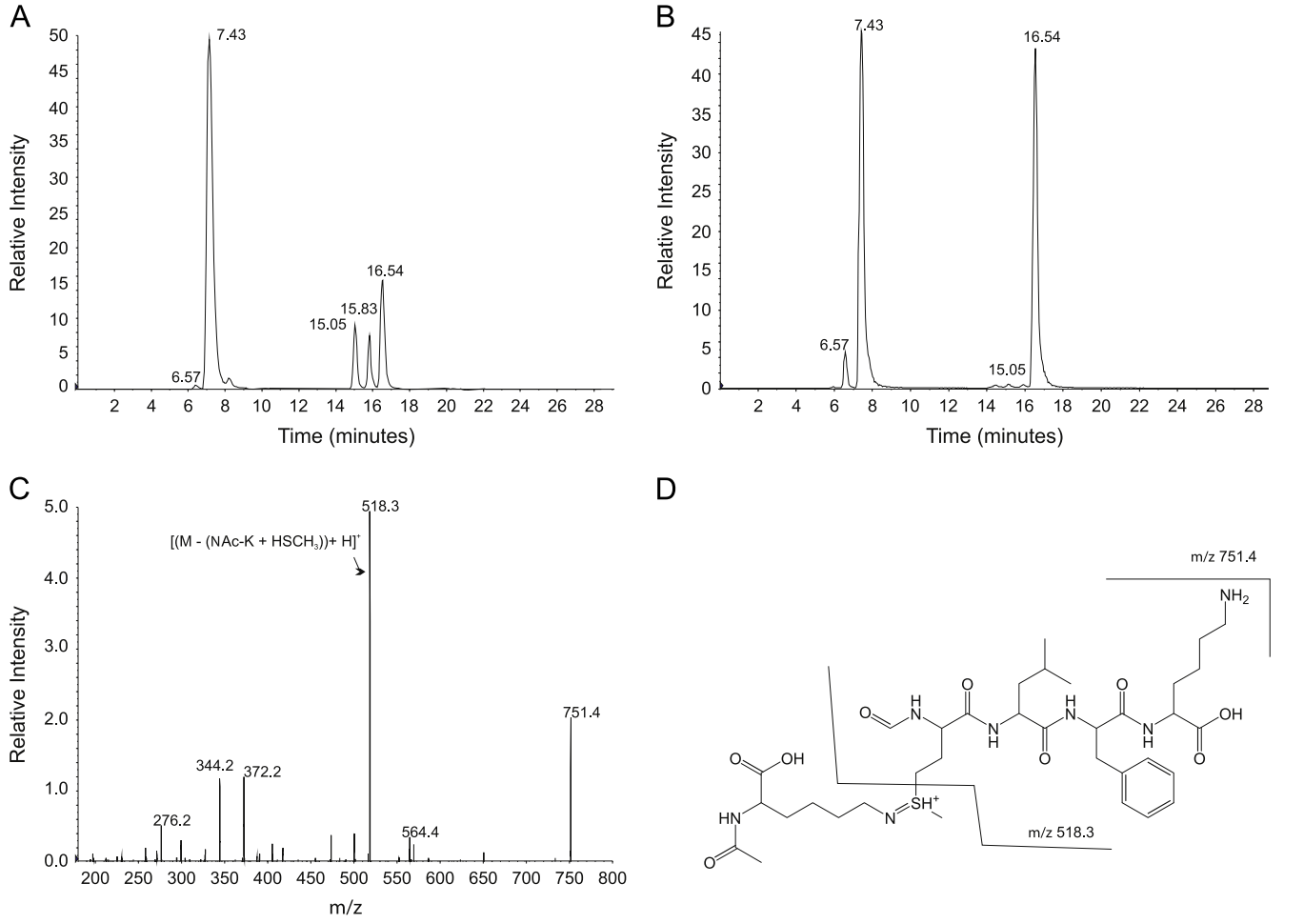

Fig. 3. Analyses of the products formed in the reaction between haloamines of N_α-acetyllysine and fMLFK. Haloamines of N_α-acetyllysine (200 μM), formed by adding hypohalous acid (200 μM) to N_α-acetyllysine, were added with vigorous mixing to fMLFK (200 μM) in 20 mM phosphate buffer, pH 7.4, containing 100 μM DTPA. Total ion count chromatograms for (A) bromination and (B) chlorination are shown. (C) MS/MS fragmentation and (D) proposed structure of *N*Ac-K-fMLFK (retention time of 6.57 min in (A) and (B)).

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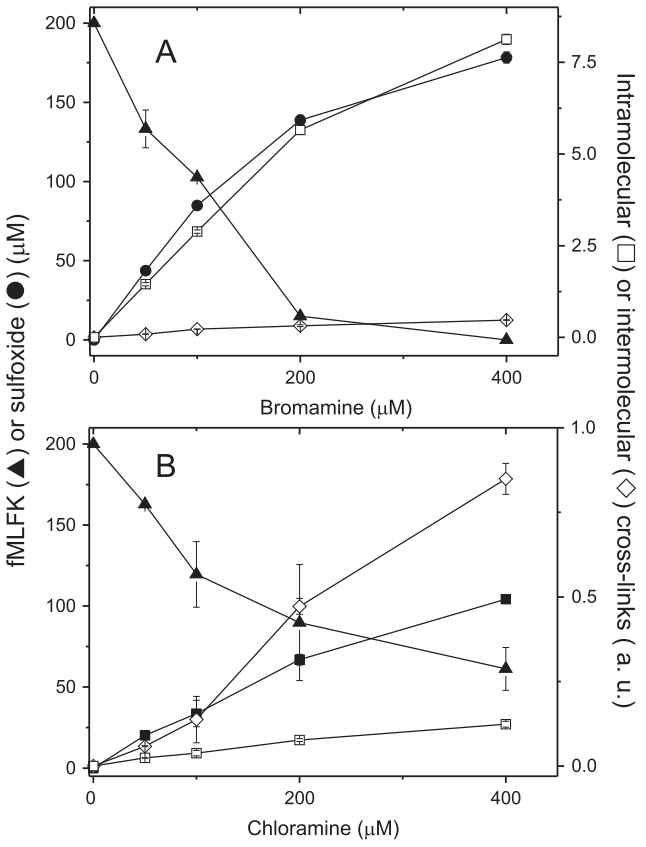

Fig. 4. The effect of the concentration of haloamines on the oxidation of fMLFK. N_α-acetyllysine haloamines (50, 100, 200, or 400 μM) were added with vigorous mixing to fMLFK (200 μM) in 20 mM phosphate buffer, pH 7.4, containing 100 μM DTPA. Reactions of (A) N_α-acetyllysine bromamine or (B) N_α-acetyllysine chloramine with fMLFK are shown. The left *y* axis shows the concentration (μM, closed symbols) of fMLFK and formation of the sulfoxide, and the right *y* axis shows the formation of the intramolecular and intermolecular cross-links (open symbols). Absolute concentrations of the peptide and sulfoxide were determined by relating their absorbance at 257 nm, due to the presence of phenylalanine, to that obtained for known concentrations of the parent peptide. Relative quantification of intermolecular and intramolecular cross-links was performed by selected reaction monitoring MS (arbitrary units, a.u.).

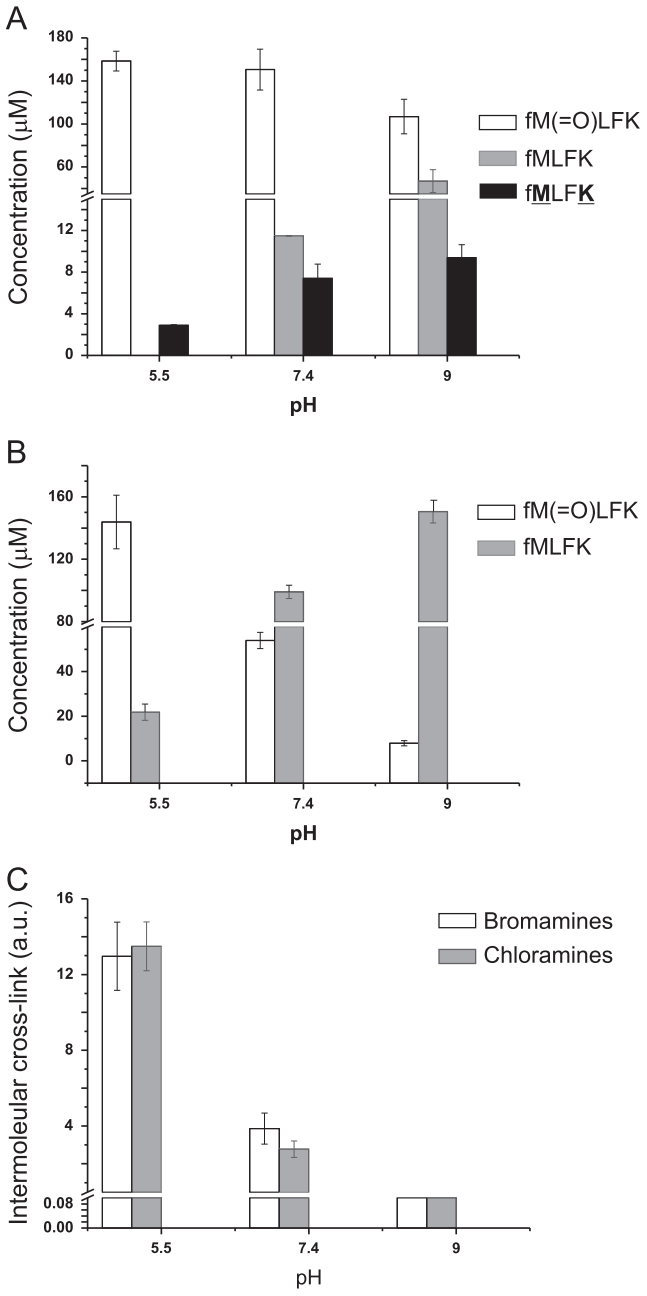

Fig. 5. The effect of pH on oxidation of fMLFK. Quantification (μM) of reactant and products generated by the reactions of (A) N_α-acetyllysine bromamine or (B) N_α-acetyllysine chloramine with fMLFK at different pH's. (C) Integration by selected reaction monitoring MS (arbitrary units, a.u.) of the intermolecular adduct generated by the reaction of N_α-acetyllysine bromamine or chloramine with fMLFK. The buffers used were citrate phosphate (pH 5.5), phosphate (pH 7.4), and carbonate (pH 9). The absolute concentrations of products in (A) and (B) were determined by relating their absorbance at 257 nm, due to the presence of phenylalanine, to that obtained for known concentrations of the parent peptide. Relative quantification of intermolecular cross-links was performed by selected reaction monitoring MS.

the intramolecular sulfilimine than the intermolecular product (Fig. 4B). Furthermore, the intramolecular cross-link was produced in far greater quantities by the bromamine than the chloramine, which favored the intermolecular cross-linked species.

The effect of pH on the reaction of fMLFK with haloamines

For both the bromamine and the chloramine, reaction of fMLFK and generation of fM(=O)LFK and *N*Ac-K-fMLFK were favored at low pH (Fig. 5). However, the yield of f**M**LF**K** increased with increasing pH, especially with the bromamine. It was not possible to evaluate the effect of pH on the generation of f**M**LF**K** by the chloramine because of its low yields.

Factors affecting product distribution

The distribution of products formed by adding hypohalous acids to a mixture of fMLFK and N_α-acetyllysine was compared with that obtained when the respective preformed haloamine was added to the peptide (Table 1). When HOCl was added to the mixture of N_α-acetyllysine and fMLFK more of the peptide reacted and more sulfoxide and intramolecular sulfilimine were produced than with preformed chloramine. However, more intermolecular cross-linked product was formed with the preformed chloramine than with HOCl. This result suggests that the sulfoxide and intramolecular cross-link share a common reaction pathway, whereas the intermolecular cross-link forms predominantly via the chloramine.

When HOBr was added directly to the mixture of N_α-acetyllysine and fMLFK the product distribution was similar to that obtained with the bromamines. The only exception was that more intermolecular cross-linked product was formed with the bromamine (Table 1). Much more intramolecular product was formed

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Table 1
Oxidation of fMLFK by hypohalous acids and haloamines in the presence of N_α-acetyllysine.

Oxidant added	Detection by UV (257 nm)			Detection by MS (a.u.)	
	fMLFK remaining (μM)	Sulfoxide (μM)	Intramolecular sulfilimine (μM)	Intramolecular sulfilimine	Intermolecular sulfilimine
HOCl	63 ± 10	103 ± 1	1.0 ± 0.1	76 ± 5	9 ± 2
RNHCl	102 ± 3	69 ± 4	< 0.5	8 ± 0.7	47 ± 5
HOBr	4 ± 2	146 ± 18	5.4 ± 0.7	648 ± 88	11 ± 2
RNHBr	14 ± 6	140 ± 5	5.4 ± 0.3	623 ± 30	38 ± 0.2

HOCl or HOBr (200 μM) was added with vigorous mixing to N_α-acetyllysine (1 mM) and fMLFK (200 μM) in 20 mM phosphate buffer, pH 7.4, containing 100 μM DTPA. The reactions were stopped after 1 min by adding methionine (1 mM). Alternatively, the hypohalous acids were initially added to N_α-acetyllysine (1 mM) to produce the corresponding haloamines (RNHCl or RNHBr) and then reacted with fMLFK. Products were quantified by relating their absorbance at 257 nm, due to the presence of phenylalanine, to that obtained for known concentrations of the parent peptide. The last two columns refer to integration by SRM MS (arbitrary units, a.u.) of intra- and intermolecular sulfimines generated in the reactions.

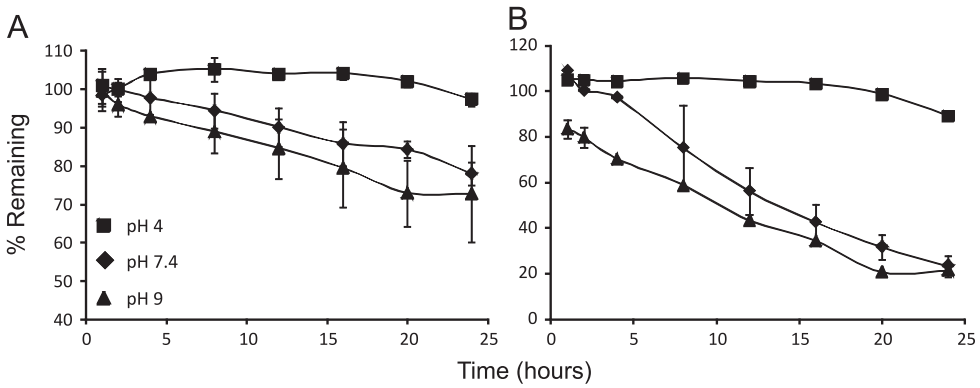

Fig. 6. The stability of (A) intra- and (B) intermolecular sulfilimine cross-links. N_α-acetyllysine bromamine (200 μM) was incubated with fMLFK (200 μM) at pH 4 (acetate buffer), 7.4 (phosphate buffer), or 9 (carbonate buffer). The adducts formed were followed by SRM MS at 37 °C for 24 h. The results are reported as the percentage of the area obtained by injection immediately after the reaction was stopped with methionine (1 mM) and were normalized by sulfoxide areas to correct evaporation through the time.

Table 2
Oxidation of fMLFK by chloramines and bromamines.

Amine	Detection by UV (257 nm)			Detection by MS (a.u.)	
	fMLFK remaining (μM)	Sulfoxide (μM)	Intramolecular sulfilimine (μM)	Intramolecular sulfilimine	Intermolecular sulfilimine
Chloramine					
N-acetyllysine	100 ± 12	71 ± 4	< 0.5	9 ± 0.5	60 ± 5.4
Glycine	63 ± 3	101 ± 2	< 0.5	4 ± 0	50 ± 2
Taurine	111 ± 18	62 ± 12	< 0.5	1 ± 0.2	84 ± 16
Ammonia	48 ± 5	130 ± 10	< 0.5	3 ± 0.3	ND
Bromamine					
N-acetyllysine	30 ± 0.7	157 ± 3	5 ± 0.1	671 ± 26	50 ± 3
Glycine	34 ± 11	135 ± 4	2 ± 0.2	367 ± 4	65 ± 4
Taurine	14 ± 2	140 ± 12	2 ± 0.2	300 ± 29	154 ± 15
Ammonia	80 ± 12	82 ± 15	0.7 ± 0.1	73 ± 17	ND

Solutions containing haloamines of N_α-acetyllysine, glycine, taurine, or ammonia were prepared by adding HOCl or HOBr to the amines as described under Materials and methods. Products were quantified as described in Table 1. The last two columns refer to integration by SRM MS (arbitrary units, a.u.) of intra- and intermolecular sulfilimines generated in the reactions. ND, not determined.

with the brominating species than with the chlorinating species regardless of the order of addition. This result also suggests that the mechanism of formation of the intermolecular cross-link by HOBr involves the bromamine intermediate and is different from that for production of the sulfoxide and intramolecular cross-link.

Stability of the cross-links

N_α-acetyllysine bromamine was incubated with fMLFK at pH 4, 7.4. and 9 and the decay of the adducts was followed at 37 °C. Both adducts were stable at pH 4 with minimal loss after 24 h (Fig. 6). The intramolecular sulfilimine was only slightly less stable at the higher pH's with losses of about 25% over 24 h (Fig. 6A). However, the intermolecular cross-link was much less stable at higher pH with only 20% remaining after 24 h (Fig. 6B).

Reaction of fMLFK with other haloamines

The chloramines and bromamines of glycine, taurine, and ammonia also reacted with fMLFK to produce inter- and intramolecular cross-linked products (Table 2). The bromamines caused more consumption of fMLFK and formation of the sulfoxide and

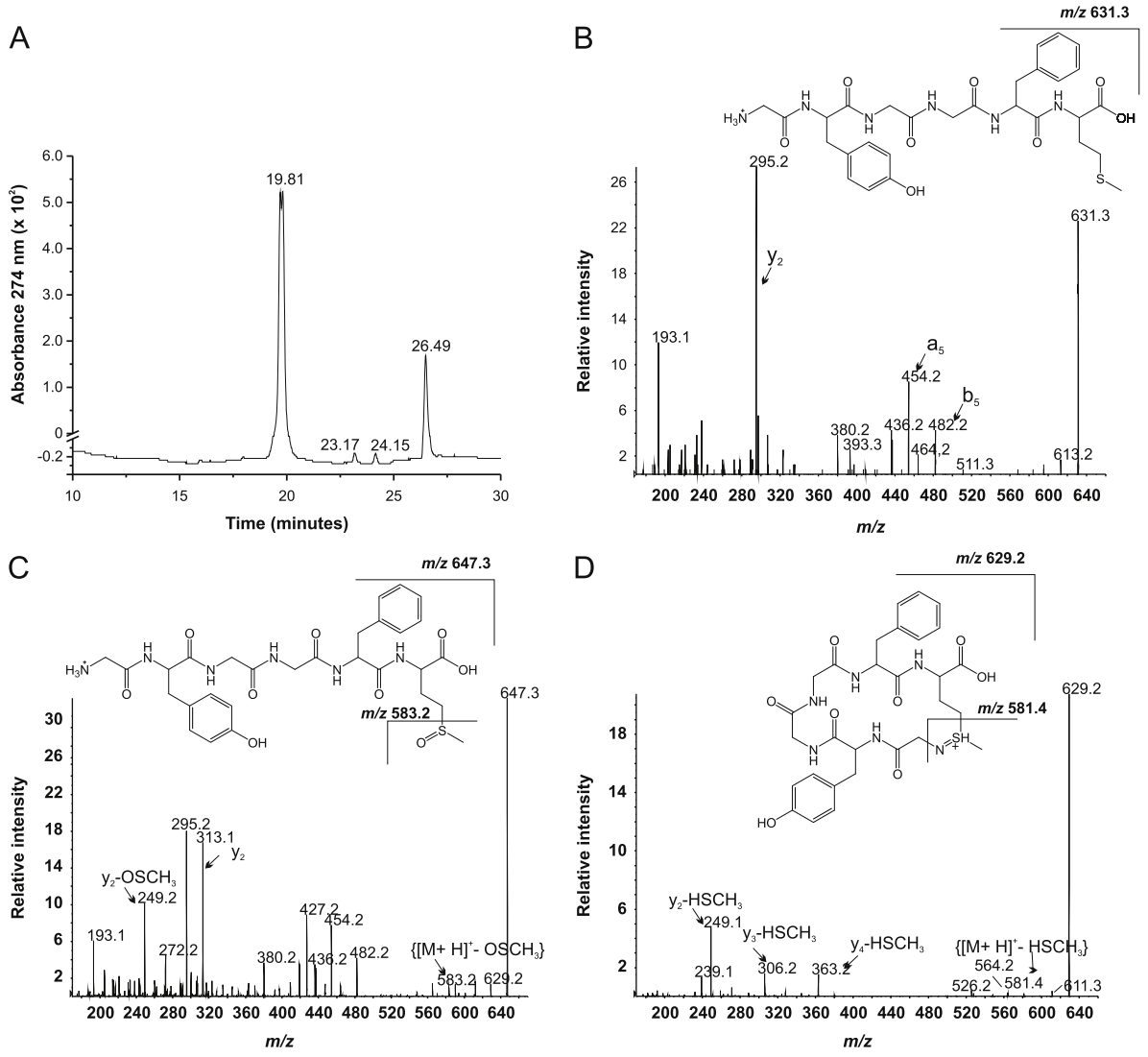

Fig. 7. LC–MS/MS analyses of the products formed in the reaction of GYGGFM (200 μM) with N_{α}-acetyllysine chloramines (200 μM). The reactions were carried out under vigorous vortexing in phosphate buffer 20 mM, 100 μM DTPA, pH 7.4, and were stopped after 1 min, by adding methionine (1 mM). (A) Chromatogram monitoring at 274 nm. (B) MS/MS fragmentation pattern for GYGGFM (retention time of 26.49 min in (A)). (C) Proposed structure and MS/MS fragmentation pattern for GYGGFM(S═O) (retention time of 19.81 min in (A)). (D) Proposed structure and MS/MS fragmentation pattern for the intramolecular adducts (retention times of 23.17 and 24.15 min in (A)).

intramolecular cross-links than their corresponding chloramines. Similar levels of the intermolecular cross-link were formed by the chloramines and bromamines. The haloamines of taurine produced the most intermolecular cross-linked products.

Sulfilimines involving an N-terminal amine

The reactions of N_{α}-acetyllysine chloramine or bromamine with GYGGFM (the *N*-terminal Gly derivative of Met-enkephalin) were investigated to determine if *N*-terminal amines can form cross-links with methionine. Fig. 7A shows an illustrative chromatogram of these reactions. The peak eluting at 26.5 min in Fig. 7A was identified as the unreacted GYGGFM (mass spectrum showed in Fig. 7B). The sulfoxide (GYGGFM(S═O), retention time of approximately 20 min in Fig. 7A) was the major product in the reaction of both haloamines with GYGGFM. Its characterization by mass spectrometry and proposed structure are displayed in Fig. 7C. Isomeric intramolecular adducts between the amino-terminal glycine and methionine (*m/z* 629.2) were identified in these reactions (retention times of approximately 23 and 24 min, Fig. 7A). Their fragmentation pattern and proposed structure are displayed in Fig. 7D. Minor amounts of an intermolecular adduct (*m/z* 816.3), corresponding to the addition of N_{α}-acetyllysine (*m/z* 187) to GYGGFM (*m/z* 631), were also observed with both the chloramine and the bromamine (data not shown).

Discussion

We have shown that HOCl, HOBr, and their respective haloamines promote intra- and intermolecular cross-links between the sulfur atom of methionyl residues and the nitrogen of amino residues. Although these sulfilimine cross-links are minor products in peptides compared to methionine sulfoxide, they are likely to be more favored at specific sites in proteins where methionine and amino residues are in close proximity. They could link residues within proteins or couple adjacent proteins together, leading to structural disorganization and aggregation. Methionine residues are favored targets of reactive halogen species. Therefore, these reactions would be expected to occur where granulocytes are stimulated, for example, in phagosomes after ingestion of bacteria and at sites of inflammation.

Hypochlorous and hypobromous acids have been shown to cause cross-linking of numerous proteins, including myoglobin

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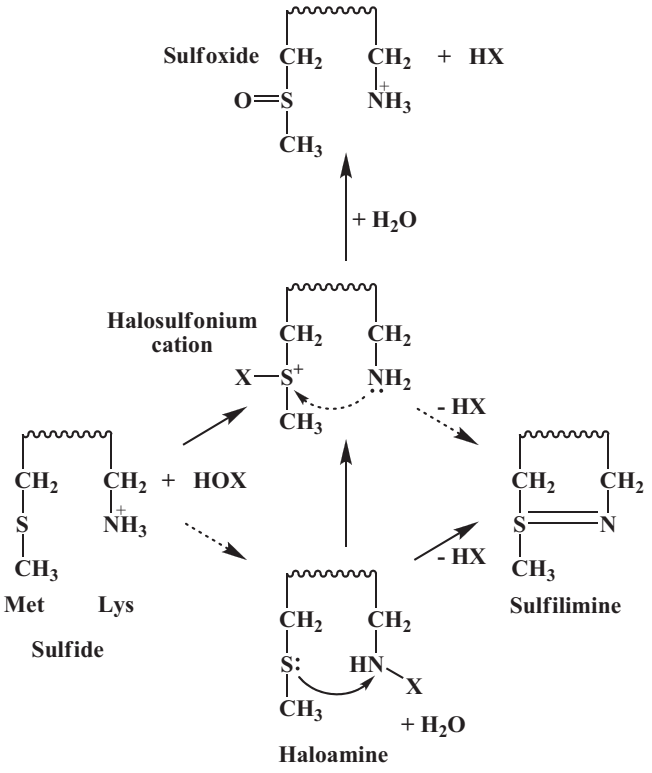
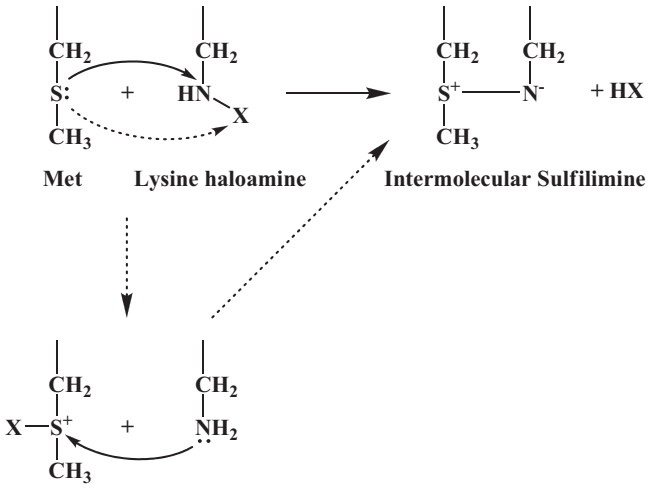


Scheme 1. Proposed reaction mechanisms for formation of intramolecular sulfilimine bonds. Hypohalous acids (HOX) react preferentially with sulfides to form halosulfonium cations but can form haloamines as minor products. The halosulfonium cations rapidly hydrolyze to produce sulfoxides. However, depending on the stability of the halosulfonium cation it can react with an adjacent amino group to produce a sulfilimine. Haloamines transfer their halogen to the sulfide or undergo nucleophilic attack by the sulfide to also produce a sulfilimine. Major reactions are shown with bold arrows.



Scheme 2. Proposed reaction mechanisms for formation of intermolecular sulfilimine bonds between haloamines and methionine. Solid and dashed arrows show alternative routes to bond formation.

[25], fibronectin [19], lysozyme [20], low-density lipoprotein [38], α_2-macroglobulin [39], and red cell membrane proteins [23]. In most cases the nature of the cross-link is not well characterized and methionine–lysine cross-linking is a plausible option. Another likely role for oxidative methionine–lysine cross-linking is as a structural component of collagen IV [30]. This sulfilimine cross-link has recently been identified and a mechanism of formation involving production of hypochlorous or hypobromous acid by the heme peroxidase, peroxidasin 1, has been proposed [10]. Our results support the feasibility of this mechanism, although further study is needed to identify the species responsible and how selectivity is achieved.

A proposed mechanism for methionine oxidation and subsequent sulfilimine formation is shown in Scheme 1. The first step in the reaction of HOCl is transfer of Cl to the sulfur atom of methionine forming a chlorosulfonium cation [14,40]. This can then hydrolyze to form the sulfoxide (the major reaction with fMLFK) or cross-link with the amine of the lysine. With fMLFK there was very little formation of the intramolecular cross-link. This contrasts with oxidation of methionine and N-terminal methionines, in which dehydromethionine was formed in abundance similar to that of methionine sulfoxide [28]. Formation of dehydromethionine is favored by the proximity of the intermediate halosulfonium cation to the amine group and the major difference with fMLFK is that these centers will be more distant. Thus, hydrolysis should be favored over ring closure (Scheme 1). This suggestion is supported by the finding that formation of the sulfoxide declined with increasing pH, whereas the intramolecular bond was optimal at higher pH. Acidic pH would prevent nucleophilic attack of the amine on the intermediate halosulfonium cation, which would allow it to hydrolyze to the sulfoxide (Scheme 1). An alternative route to the sulfilimine is via chloramine formation on the lysine residue, but this is less likely to be involved because HOCl reacts 4 orders of magnitude faster with methionine than with lysine [16].

The intramolecular cross-link with HOBr is likely to form via the analogous bromosulfonium cation. However, the alternative pathway via bromamine formation could also be involved in the cross-linking reactions. This is because HOBr reacts only 10-fold faster with the methionyl residue than the amino group of lysine [12], allowing some bromamine formation along with the bromosulfonium cation (Scheme 1). Thus cross-linking could occur by coupling of the amine with the bromosulfonium cation or via attachment of the sulfide to the bromamine (Scheme 1) [41]. This, as well as the greater stability of the bromosulfonium cation toward hydrolysis as reported previously [28,29,42], could explain the greater efficiency of hypobromous acid compared with hypochlorous acid.

Bromamines were as effective as HOBr at forming the intramolecular cross-link. This finding is explained by the facile transfer reactions between free amines and bromamines, which occur more readily than with the analogous chloramines [43]. Thus, the distribution among bromamines in a mixture will be determined more by equilibrium consideration rather than by the relative rates of formation, which determine the distribution of chloramines [43]. Hence, the intramolecular cross-link could potentially also form via the bromamine on the lysine residue.

In contrast to the intramolecular cross-link, the intermolecular linkages promoted by chloramines and bromamines most likely occur via nucleophilic attack of the sulfide of methionyl residues rather than via halide transfer to the sulfide (Scheme 2). This mechanism is supported by our finding that intermolecular cross-links were favored when methionine-containing peptides reacted directly with haloamines rather than when the hypohalous acids were added to a mixture of amines and peptide. Also, the finding that intermolecular cross-links were formed to a similar extent by analogous chloramines and bromamines supports our contention that halosulfonium cations were not involved in this process. Otherwise yields of the cross-links would reflect differences in stability of these two intermediates. The favorable formation of intermolecular cross-links at low pH suggests that protonation of the haloamines is likely to favor their reaction with sulfides.

The second-order rate constants for oxidation of methionine by glycine chloramine, *N*-acetyllysine chloramine, and taurine

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chloramine are 197, 52, and 39 M^{-1} s^{-1}, respectively [44]. It was, therefore, interesting to find that the taurine haloamines, which exhibit the lower reactivity toward the sulfur group, generated the highest yield of intermolecular adducts, but the lowest yields of f**M**LF**K**. This is possibly because they are poor at transferring the halogen to the sulfide but suitably stable to allow nucleophilic attack by the sulfide.

Our results indicate that sulfilimine bonds formed between methionyl and amino residue are very stable at physiological pH, taking hours to decay. The intramolecular bond in f**M**LF**K** was more stable than its intermolecular bond with N_{α}-acetyllysine. This suggests that sulfilimine bonds formed within proteins in vivo should be stable and will be readily detected as witnessed by identification of the cross-links in collagen IV [10,45].

In summary, our studies have shown that reactive halogen species promote the formation of intra- and intermolecular adducts between methionine and amine groups. Formation of these adducts will be in competition with sulfoxide generation, but their production will be favored in proteins when methionine and lysine residues are juxtaposed and poised for coupling. In collagen IV, for example, a sulfilimine bond is apparently formed at a specific site with high efficiency. Hypohalous acids may not be the only physiological oxidants that generate these cross-links, as oxidation of methionine residues by singlet oxygen and hydroxyl radical can generate similar products [14,46–49]. The generation of intramolecular and intermolecular adducts between cysteine residues and amine groups in proteins by hypochlorous acid has raised the possibility that these adducts contribute to protein cross-links in the artery wall as well as in other inflammatory conditions [50]. Methionine is more abundant than cysteine in proteins. Thus, sulfilimine adducts are also likely to contribute to oxidative cross-linking and their involvement in tissue damage associated with inflammation and other pathologies warrants further research.

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