Oxidative modification of ovalbumin*

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Stimulated neutrophils (PMNL) are a source of the active oxygen species: O₂, H₂O₂ and HOCl/OCl⁻ which in turn can act on proteins yielding a variety of oxidative products. A system is proposed in which a model protein — ovalbumin (OVA) first undergoes chlorination by HOCl/OCl⁻ and next is oxidised by H₂O₂. The modification of functional groups (–NH₂, –SH, –S–S–, →C=O, Tyr and Trp) in OVA was monitored as well as their accessibility to promote aggregation. Chlorination resulted in additional inter- or intra-S-S-bond formation followed by a decrease in the total sulphydryl group content. Amino groups were oxidised to carbonyl moieties with a concomitant acidic shift of pI. Formation of chlorotyrosine at the chlorination step was confirmed and its further H₂O₂-mediated transformation to bityrosine was demonstrated. It has also been confirmed that tryptophan, and not tyrosine, is the first target for chlorination. SDS/PAGE and HPLC profiles revealed that HOCl/OCl⁻ chlorination promotes formation of aggregates stabilised by non covalent bonds. In conclusion, we suggest that a dramatic change in the OVA molecule structure begins when the molar excess of HOCl/OCl⁻ is about 2 per one reactive group in OVA.

In recent years, much evidence has accumulated that HOCl, the major neutrophil originating oxidant [1–4] reacts with various biological molecules [5–9]. Hypochlorous acid, however, is a product of the myeloperoxidase system which requires H₂O₂ as a substrate. When stimulated, neutrophils undergo a respiratory burst during which oxygen is converted to the superoxide (O₂⁻) which dismutates to H₂O₂ [1, 2]. Thus, three major oxidants (HOCl/OCl⁻, H₂O₂, O₂⁻) can react with biomolecules yielding a variety of oxidation products [10]. Proteins appeared to be very important targets for HOCl/OCl⁻; their modification leads to: loss of biological function [11], enhanced proneness to proteolytic degradation [12], mammalian cell injury [13], bacteria killing [14] and increase in immunogenic properties [15, 16].

Since in 1975 Zgliczyński & Stelmaszyńska [3] found that bovine serum albumin is chlorinated by the myeloperoxidase-H₂O₂-Cl⁻ system, changes in the structure of some proteins

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Abbreviations: OVA, ovalbumin, PMNL, polymorphonuclear leukocytes, neutrophils; RFI, relative fluorescence intensity; TNBS, 2,4,6-trinitrobenzenesulfonic acid, DTNB, 5,5'-dithiobis(2-nitrobenzoic) acid, DTT, D,L-dithiothreitol.
modified by HOCl/OCI⁻ or by the chlorinating myeloperoxidase system were described [9, 17–19].

During the respiratory burst the relative intensities of chlorination or oxidation depend on local concentration of H₂O₂ and HOCl/OCI⁻. Since HOCl/OCI⁻ reacts more rapidly with functional groups of proteins than H₂O₂ and forms the long-lived protein chloramines it seems probable that the chlorinated protein can undergo further oxidation by O₂ or H₂O₂ as long as activated neutrophils are present at the inflammatory focus.

The present studies were undertaken to analyse the changes in the structure of protein chlorinated in mild conditions to establish the kind of chemical changes the protein undergoes in the absence of oxidants other than HOCl. Further oxidation, however, was provoked by addition of H₂O₂ to the previously chlorinated protein. Thus, this way of oxidation may mimic the multi-step oxidation process which can occur in the inflammatory locus. Ovalbumin has been chosen as the target protein because its structure is well known [20–22], and because our recent studies revealed that ovalbumin chlorinated by HOCl/OCI⁻ is much more immunogenic than the native protein [15].

MATERIALS AND METHODS

Ovalbumin grade V, bis-acrylamide, Coomassie brilliant blue R and D,L-dithiothreitol were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Acrylamide, dodecyl sulfate Na salt (SDS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were from Serva Feinbiochemica (Heidelberg, Germany). N,N,N',N''-tetramethyl-ethylenediamine (TEMED), taurine, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and Tris were from Fluka Chemie AG (Switzerland). 2,4-Dinitrophenylhydrazine was from Merck (Darmstadt, Germany), and ammonium persulfate from Reanal (Budapest, Hungary). Sodium phosphate-monobasic monohydrate, sodium phosphate-dibasic heptahydrate, sodium hypochlorite and hydrogen peroxide were from Aldrich Chemie (Steinheim, Germany). NaOCl solutions were prepared prior to use from 5% stock solution and were standardised iodometrically [23, 24]. H₂O₂ solutions were prepared from 30% H₂O₂ and standardised manganometrically. The other reagents of analytical grade were from P.O.Ch. (Poland).

Spectrophotometric measurements were carried out with a UV-VIS Spectrophotometer Gilford (Ohio, U.S.A.) and with a Hitachi U-2000 Spectrophotometer (Tokyo, Japan). Fluorescence spectra were recorded with a Hitachi F-2000 fluorescence spectrophotometer (Tokyo, Japan).

Protein chlorination. Ovalbumin at a concentration of 2 mg/ml in 0.05 M phosphate buffer, pH 7.4, was dialysed against outer 0.05 M phosphate buffer containing NaOCl at initial concentrations ranging from 0.065 to 0.95 mM. The volume of the outer buffer was adjusted so as to attain the required ratio of the total hypochlorite to the protein. Dialysis lasted for 48 h at 4°C. The samples after chlorination were treated with thiosulfate at the molar ratio of 2:1. The excess of thiosulfate was dialysed off against outer 0.05 M phosphate buffer, pH 7.4, for 24 h at 4°C. Thiosulfate at the concentrations used did not interfere with determination of amino groups by the TNBS method [25].

Chlorination of model N-acetyl-tyrosine and N-acetyl-tryptophan. Samples contained 16 μmol of an amino-acid derivative and 80 μmol of HOCl/OCI⁻ in 4.2 ml of 0.2 M phosphate buffer (pH 7.4). After 20 min incubation, 80 μmol of thiosulfate was added to stop the reaction. Control samples contained all the reagents except HOCl/OCI⁻. The difference spectra of chlorinated amino-acid derivatives versus unchlorinated ones at the final concentration of 3.1 mM in 1% SDS at pH 7.4 and after acidification to pH 2.5 were obtained.

Hydrogen peroxide oxidation of chlorinated OVA. Samples of chlorinated OVA (2 mg/ml) were incubated with 30 mM hydrogen peroxide for 168 h. The H₂O₂ concentration was increased to 60 mM after 96 h of incubation. Fluorescence emission spectra were recorded until the 166th hour of incubation at the excitation wavelength of 310 nm.

Monitoring amino groups in OVA. Modification of chlorinated proteins was traced in the change of free amino group content measured by the TNBS method [25]. The samples containing 1.2 mg of unmodified or chlorinated ovalbumin and 0.6 mM TNBS in 1.6 ml of 2.5 mM borate buffer, pH 9.2, were incubated for 30 min at 37°C. Next, 1.2 ml of 0.2 M NaH₂PO₄ con-
Oxidative modification of ovalbumin

Vol. 43

containing 18 mM Na₂SO₃ and 0.5 ml 2.5% SDS were added, and after 2 h incubation at room temperature the absorbance at 420 nm was monitored. The concentration of -NH₂ groups was calculated assuming ε₁₂₀ = 13.0 M⁻¹ cm⁻¹ [25].

Assays of carbonyl group content. The carbonyl content was determined after reaction with dinitrophenylhydrazine according to Oliver [26]. Assays were carried out on 0.2 ml samples of native and chlorinated OVA (0.4 mg) and concentration of the protein dinitrophenylhydrazones was calculated using the millimolar absorption coefficient of ε₅₆₅ = 21 mM⁻¹ cm⁻¹.

Measurement of sulfhydryl and disulfide levels. Assays were carried out with the use of DTNB with or without dithiothreitol (DTT) according to Kitabatake & Doi [27]. In the disulfide assay the DTT-reduced, native and chlorinated samples were fractionated on a Bio-Gel P-2 column (1.2 cm i.d. × 30 cm height) and DTNB was immediately added to 1 ml fractions of the eluates to measure the sulfhydryl levels. Ovalbumin content in fractions were calculated from the absorbance at 280 nm based on the value of A₄₃₅₉ = 7.12 [28] and sulfhydryl levels, on ε₄₁₂ = 13.6 M⁻¹ cm⁻¹ [29].

Estimation of dichlorotyrosine content. The number of dichlorotyrosine groups per OVA molecule were calculated from difference spectra of chlorinated OVA preparations using ε₃₀₅ = 4.7 M⁻¹ cm⁻¹ [30].

Estimation of bityrosoine and tryptophan contents. The amount of bityrosoine was calculated from difference spectra between chlorinated OVA and chlorinated OVA incubated with an access of hydrogen peroxide assuming the millimolar absorption coefficient of ε₁₅₅ = 32.8 M⁻¹ cm⁻¹ [31]. The calculated amounts were correlated with the relative fluorescence intensity (RFI) for emission at 410 nm (excitation wavelength: 310 nm). The number of tryptophan residues was calculated from RFI at 380 nm for two excitation wavelengths: 274 and 310 nm.

HPLC assays. Size exclusion chromatography was performed in a TSK G2000 SW column (7.5 mm × 300 mm) or in G2000 SW and G23000 (7.5 mm × 300 mm) SW columns in line using the KONTRON HPLC system. Samples containing 1–3 μg of protein were injected. Elution (0.5 ml/min) was carried out with 0.1 M phosphate buffer (pH 7.2) containing 0.3 M sodium chloride, and monitored at 220 nm.

Lysozyme (14.3 kDa), trypsin (23 kDa), bovine serum albumin (67 kDa) and native human prostatic acid phosphatase (100 kDa) were used as standard proteins for column calibration. HPLC assays were done with chlorinated OVA samples or OVA samples additionally preincubated with 2% DTT for 24 h.

SDS/PAGE. Slab gels of 10% acrylamide were developed with Coomassie blue by the standard staining procedure. Lanes were loaded with 5 μg of protein after denaturation for 15 min at 100°C in SDS containing buffer in the presence or absence of 3% mercaptoethanol.

Isoelectrofocusing profiles. These were obtained with the use of horizontal DEAE-agarose (Desaga, Heidelberg) at 1.5 W constant power for 5 h at 5°C. The cast gels were 150 μm Servalyt Precoats 3–10. The anolyte and catholyte were phosphoric acid and sodium hydroxide, respectively, 1 M each, with the interwick distance of 10 cm between the cathode and anode wick edges. Gels were developed by the Coomassie blue standard procedure.

RESULTS

Functional group modification in OVA

Native ovalbumin contains four sulfhydryl groups and one disulfide bridge per molecule [20]. Chlorination with hypochlorite decreases the number of sulfhydryl moieties in OVA. As one can see in Fig. 1, the difference between the sulfhydryl level in unreduced and DTT-reduced ovalbumin corresponds to the average number of cysteinyl moieties involved in the -S-S- bridge formation. Since the content of sulfhydryl groups decreases with the increasing molar ratio of HOCl to ovalbumin, it can be stated that -SH moieties react rapidly with the chlorinating agent. As shown in Fig. 1, at HOCl concentration of 2.5 μmol/μg, an OVA molecule has on average only 0.5 intact -SH group and 1.5 -S-S- bond. Since the native molecule contains only one -S-S- bridge it can be concluded that chlorination results in additional inter or intra -S-S- bond formation. Simultaneously, further oxidation must take place and lead to products which cannot be detected by the DTTB method. The amount of these products — probably cysteinyl residues [7] — can be estimated as the difference between the total content of the sulfhydryl moieties in the native
and the chlorinated samples and, as it is apparent from the data presented in Fig. 1, their level increases with increasing HOCl/OVA ratio.

Chlorination of amino groups in proteins leads to chloramine formation [17]. In the presence of an excess of hypochlorite the chloramine is oxidised to an aldehyde or carboxylic moiety. Cross-linking Schiff bases can be formed between carbonyls and unreacted protein amino groups [17]. Chlorination through membrane is a relatively slow process due to slow diffusion of the reagent (HOCl/OCl⁻) through the membrane. Once formed chloramines exist in the mixture at least for 48 h, after that time thiosulfate was added to transform them to amino groups. Since during the reaction a part of the protein N-chloramines underwent further oxidation to carbonyl compounds, the loss of amino groups can serve as a measure of transformed N-chloramine groups. This loss became significant when the HOCl concentration was greater than 3 μmol/mg OVA (Fig. 2). The loss of 1.5–2 -NH₂ groups per mole of OVA corresponds to formation of an equal amount of carbonyl groups in OVA (Fig. 2). This seems to be the only path of carbonyl formation in the preparations. Moreover, it can be concluded that, over a broad range of HOCl/OCl⁻ concentrations (0–4 μmol/mg OVA), the local concentration of HOCl/OCl⁻ in the system did not increase since level of amino and carbonyl groups was almost the same (Fig. 2), whereas in the reactions carried out in situ, an excess of HOCl/OCl⁻ caused an increase in the carbonyl group content [8, 12]. It is interesting that not more than 50% of the amino groups exposed in native form was accessible to HOCl/OCl⁻ even at its high concentration (Fig. 2).

Tryptophan and tyrosine are well known targets for chlorination [7, 18]. The absorption shown by their chlorinated derivatives contributes to the observed absorbance in the range of 300–310 nm in the spectra of the chlorinated OVA. In general, however, it is still unclear which product of the amino-acid chlorination dominates in the protein. In the case of OVA the difference spectra reveal the presence of a peak at 306–307 nm at pH 7.4 (not shown) which could be attributed to the either of the oxidation product since OVA contains 3 tryptophan and 10 tyrosine residues [20]. The loss of the tryptophan moieties, however, could be estimated from the decrease of the fluorescence emission at 350 nm at excitation wavelength 274 nm (Fig. 3). As one can see in the Fig. 3 the fluorescence of the sample drops to 0 at 2 μmol of HOCl. Thus, the absorbance of this sample at 306 nm is the measure of the maximal content of chlorinated Trp. Consequently, the
Further increase of absorbance observed in OVA samples containing HOCl/OCl⁻ at higher concentrations has to result from formation of Tyr chloroderivative. It can be estimated that the real amount of dichlorotyrosine at higher HOCl concentrations (2–6 μmol/mg OVA) does not exceed 3–4 per one OVA molecule. Furthermore, the absorption maximum at 306 nm, not at 315 nm [31], confirms that mainly dichlorotyrosine, not bityrosine, is formed during the slow chlorination through membrane.

Lowering of the pH down to 2.7 caused a dramatic drop of the absorption at 306 nm for samples of OVA chlorinated with HOCl (in the range of 2–6 μmol/mg OVA), which resembled changes occurring in spectra on acidification of chlorinated N-acetyl-Tyr, but not N-acetyl-Trp (not shown). This further supports the idea that, in the system studied mainly 3,5-dichloro-
tyrosine is formed in OVA at higher HOCI/OCI⁻ concentrations.

To establish whether, and if — how many tyrosine moieties can be converted to 3,3'-bityrosine crosslinkages, fluorescence measurements were carried out and emission spectra excited at 310 nm were recorded. The results summarised in Figs. 4–6 reveal that the chlorination itself does not lead to a massive bityrosine formation as it has been observed for other proteins in systems containing hydrogen peroxide [9, 32]. However, long incubation with H₂O₂ resulted in conversion of all dichlorotyrosines and unexposed tyrosines present in OVA to crosslinked products. As one can see in Fig. 4, all 10 tyrosine moieties were present in the crosslinked form in the samples previously chlorinated at higher (4–6 μmol/mg) HOCI/OCI⁻ concentrations. Moreover, the formation of bityrosine moieties upon H₂O₂ action was confirmed by an increase of the absorption at 315 nm observed at pH 7.4 (not shown). A more detailed analysis of the shifts of the fluorescence maxima revealed that the spectra observed for samples chlorinated with high doses of HOCI/OCI⁻, showed red shifted maxima as compared with those predicted for pure bityrosine and, moreover, the maxima were shifted towards the shorter wavelengths during the incubation with hydrogen peroxide (Fig. 5, 6). This leads to the conclusion that originally (along with small amounts of bityrosine) also the other fluorescent products were formed at the chlorination step. It has been shown that oxidised tryptophan in proteins exhibits a fluorescence peak at 450 nm which could be attributed to the kynurenine moiety [33, 34]. It has been also observed that chlorination of N-acetyl-tryptophan results in the appearance of fluorescence at 438 nm (not shown). Therefore, the red shifted maxima observed for chlorinated samples of OVA could have originated from kynurenine fluorescence and, consequently, the blue shift following the reaction with hydrogen peroxide, resulted from their slow decomposition.

The relative amount of bityrosine moieties formed during the incubation with H₂O₂ increased with the degree of “priming chlorination”, probably due to an HOCI/OCI⁻ mediated unfolding process. The question arises whether the bityrosine moieties are formed solely
Fig. 5. The fluorescence emission spectra of ovalbumin prior to the A, H$_2$O$_2$ addition and B, after 166 h incubation with H$_2$O$_2$.

Samples contained 1, 15 μmol of H$_2$O$_2$ and 1 mg of native OVA, or OVA chlorinated with HOCl doses of: 2, 2.5; 3, 3.5; 4, 4.5 μmol/mg OVA. Spectra were recorded using 310 nm excitation wavelength.

Fig. 6. Changes of the fluorescence emission peak maxima in ovalbumin exposed to H$_2$O$_2$.

Samples contained ○, native; or △, ×, ■, chlorinated OVA. The amounts of all reactants as for Fig. 5. Spectra recorded using 274 nm excitation wavelength.
Fig. 7. SDS/PAGE profiles of ovalbumin exposed to HOCl.
Gels were developed with Coomassie blue. Lanes refer to 1. native and chlorinated OVA with the following HOCl doses: 2, 2.5, 3, 4, 5, 6, 7.5 μmol/mg OVA. Only the section of the gel containing OVA is shown, with the position of 45 kDa and 12.5 kDa indicated by arrows.

Fig. 8. HPLC profiles of chlorinated ovalbumin after incubation with DTT.
Native OVA without A, DTT or chlorinated with HOCl doses: B, 0 μmol/mg OVA. For other details see “Materials and Methods”.
from intact tyrosine in OVA or also via its chloroderivatives. The calculations of the bityrosine content based on absorbance at 315 nm indicate that such a path is also possible since out of five bityrosine crosslinkages present in maximally chlorinated samples at least two could be formed via tyrosine chloroderivatives. This seems to be in agreement with the observation that the number of intact tyrosines in the maximally chlorinated OVA did not exceed six (Fig. 4).

**OVA SDS/PAGE and HPLC profiles**

SDS/PAGE profiles of the chlorinated OVA run in reducing conditions show that increasing HOCI concentration led to a limited aggregation of OVA and its partial fragmentation (Fig. 7). HPLC results indicated, however, that before being reduced by the dithiothreitol OVA existed mainly as a monomeric protein with the molecular mass close to that of the native one over a broad range of HOCI/OCI⁻ concentrations (peaks — 18 min and 17 min retention time, respectively, for native and chlorinated OVA, 4.5 μmol HOCI/mg OVA — not shown). It was also found that, at the HOCI/OCI⁻ concentration higher than 2.5 μmol/mg, OVA existed mainly as a dimer or migrated as a partially unfolded molecule. Moreover, the SDS/PAGE profiles in non-reducing conditions did not point to the existence of a stable dimer (not shown). Therefore the peak of 17 min would not represent the stable dimer stabilised with -S-S- but could be attributed either to a dimeric form stabilised by weak interactions, or to the partially unfolded monomer. Neither it is possible to conclude from the HPLC data alone whether the dimer is formed from an intact OVA monomer or, as pointed to by the SDS/PAGE data, also from OVA fragments of lower molecular mass resulting from fragmentation. A small amount of aggregation products appearing on SDS/PAGE above the main band (Fig. 7), stabilised by a covalent bond different from the -S-S- group, could be attributed to bityrosine linkages.

**Chlorination promotes OVA aggregation**

As indicated by the HPLC profile (Fig. 8), both HOCI chlorinated and unchlorinated OVA aggregate when they are incubated with a bifunctional reductant DTT (and, to some extent, with a monofunctional one, mercaptoethanol — not shown). The HPLC profiles show higher peaks (at 12 min and 14–15 min) for the chlorinated samples than for the native ones over the whole HOCI/OCI⁻ range applied (Fig. 9). The peak at 12 min can be attributed to a high molecular species (molecular mass 600 kDa) whereas the peak at 14–15 min to a dimer or trimer. As one can see, the accessibility to aggregation was the most pronounced in the concentration range of 2–4 μmol HOCI per 1 mg OVA. The content of
small aggregates, however, increased with the HOCI/OCl− concentration. The aggregate formation proceeded effectively only in the case of chlorinated OVA. It looks that HOCI/OCl− excess increases someway the weak interactions between partially unfolded OVA molecules. This explanation is further supported by the fact that the aggregates were also formed in the solutions which did not contain SDS.

DISCUSSION

X-Ray crystallographic studies show that all three tryptophan moieties are situated in the interior of the ovalbumin molecule [21]. Despite the fact that two of them, Trp146 and Trp267, are included in the helices they are accessible to external agents and display altered fluorescence due to the reversible unfolding processes [22]. Our data confirm their accessibility since very minute doses of HOCI/OCl− convert them to nonfluorescent derivatives. As tryptophan, and not tyrosine, seems to be the first target for chlorination, this would suggest that at least a part of tyrosines are buried within the ovalbumin globule in a such way that their residues could only be chlorinated after extensive unfolding had occurred. The total number of tyrosine moieties which were chlorinated in our system did not exceed 4–6 out of 10. This suggests that at least 4 of them could be situated in an inner part of the globule. This result correlates with X-ray data [21] which reveal that 9 out of 10 tyrosine moieties are situated in helical fragments which are resistant to unfolding agents [22]. It has been established [9, 32] that formation of 3,3′-bityrosine linkages in oxidised proteins depends on the number of accessible surface tyrosines. Hydrogen peroxide or peroxidase systems are known mediators of bityrosine formation. Also HOCI/OCl− alone used in excess is able to oxidise tyrosine to bityrosine, as it was suggested by Vissers & Winterbourn [9]. However, the formation of dichlorotyrosine as well as detection of 3-chlorotyrosine in pronase digests of chlorinated human albumin [18] strongly suggest that formation of chloro- and not bityrosine derivatives is preferred in this system. The best explanation of this fact, according to our results, is that there is no local excess of HOCI/OCl− in the reaction mixture because of the diffusion limited influx of the reagent through the membrane. Moreover, a great excess of H2O2 and prolonged incubation time as well, are necessary to transform intact tyrosines and their dichloroderivatives to bityrosine moieties. This suggests that, in physiological conditions, the proteins chlorinated by HOCI/OCl− or chloramines could be slowly transformed to the aggregated final product during contact with a second oxidant (e.g. H2O2).

As it was shown earlier [12, 17] the protein chloramines slowly decompose to carbonyl moieties. This change would result in an increase in the acidic character of the protein. As the isoelectrofocusing profiles show (Fig. 10), decomposition of a basic amino group is followed by an acidic shift of pl of the chlorinated OVA samples. It could be also seen that the shift correlates with the loss of the amino groups and becomes very significant at HOCI/OCl− concentrations greater than 3 μmol/mg OVA (Fig. 2 and 10).

Slow chlorination did not lead to massive formation of bityrosine linkages although small amounts of covalent aggregates could be seen on gels (Fig. 7). Their minute content resulted, as well, in weak fluorescence emission at 410 nm (Fig. 4). The observed tendency to

Fig. 10. Isoelectrofocusing profiles of ovalbumin exposed to HOCI.
Gels were developed with Coomassie blue. Lanes refer to native, 1; and chlorinated OVA with the following HOCI doses of: 1, 0; 2, 1; 3, 2.5; 4, 4.5; 5, 6 μmol/mg OVA.
formation of dimers and aggregates, especially in the presence of DTT suggests non covalent character of bonding since gels run in non reducing conditions did not show bands of molecular mass higher than 45 kDa (not shown). The weak non covalent character of the interactions is confirmed by the fact that aggregates formed in the presence of DTT are not formed, or decompose, when SDS is present. On the other hand, a small increase in the number of -S-S-bonds (Fig. 1) following chlorination could be attributed to formation of intra - S-S- linkages within the OVA molecule.

All these observations suggest that more dramatic structural changes begin at a HOCl/OCl⁻ of range 2–3 µmol per 1 mg OVA as was shown under Results (Figs 1, 2, 4, 8). This HOCl concentration provides its molar excess of 40–50 mol per mol of OVA. Taking into account the whole number of the HOCl/OCl⁻ accessible, highly reactive groups in OVA (16 Met, 4 -SH, 6 Lys, 3 Trp, not taking into account Tyr) [20] and assuming the average HOCl/OCl⁻ consumption per group to be 1–2 [7] it can be calculated that the resulting molar HOCl excess is about 2 per one reactive group in the case of 2–3 µmol HOCl/mg OVA concentration range. This assumption supports the idea that during chlorination through the membrane this process occurs stepwise and does not provide the system with a local HOCl/OCl⁻ excess. Thus in our system there are no HOCl/OCl⁻ molecules playing the role of the second oxidant.

Proteins which are damaged by oxidation/ chlorination become deprived of their biological function, especially when the effects of modification are pronounced. In fact, such proteins are more susceptible to proteolysis [9, 12], so they are destined to undergo degradation. Moreover, the HOCl-modified proteins exhibited enhanced immunogenic properties in vitro [15, 16]. The ovalbumin chlorinated under conditions described in this paper was not only more efficiently processed by the A-20-2J (APC) cells, but also its presentation as an antigen to T-cells was improved [15]. It is striking that the highest immunogenic effect was found in the case of ovalbumin chlorinated with 2–7 µmol HOCl/mg of protein [15], i.e. the range of HOCl/OCl⁻ concentration at which ovalbumin undergoes dramatic structural changes.

REFERENCES


