INTRODUCTION

Influenza virus is an enveloped, negative-sense ssRNA virus with three transmembrane proteins, two of which are known as spike proteins, the haemagglutinin (HA) (Gamblin & Skehel, 2010; Skehel & Wiley, 2000) and neuraminidase (NA) (Xie et al., 2011). The functions of HA are indispensable for the early establishment of infection in a target cell (Skehel & Wiley, 2000). An HA receptor on the target cell binds to a receptor-binding site of HA. The target cell (Skehel & Wiley, 2000). An HA receptor on a target cell binds to a receptor-binding site of HA. The receptor-binding site just below the acetamide moiety of the sialic acid (N-acetyleneuraminic acid) residue of the receptor (Lin et al., 2007). Conserved amino acid residues are present in the receptor-binding site, including Y98, S136, W153, H183, E190 and Y195 (Gamblin & Skehel, 2010; Stevens et al., 2004, 2006). Among these conserved residues, W153 is located at the bottom of the binding site just below the acetamide moiety of the sialic acid (N-acetyleneuraminic acid) residue of the receptor (Lin et al., 2009).

Chlorine dioxide (ClO₂) is a relatively stable free radical. ClO₂ can inactivate various bacteria (Morino et al., 2011), fungi (Morino et al., 2007) and viruses (Morino et al., 2009; Sanekata et al., 2010). Ogata & Shibata (2008) recently reported a 6-month continuous inhalation experiment using rats in which they showed that a 0.05 p.p.m. level of ClO₂ had no adverse effect. The effectiveness (Ogata & Shibata, 2008) of low-concentration ClO₂ gas indicates that this might be useful in preventing the transmission of influenza virus (Ogata & Shibata, 2009). Although Ogata & Shibata (2008) showed that ClO₂ inactivated influenza virus, the molecular details of this inactivation mechanism were unclear. The results presented in this paper clearly demonstrate that inactivation of influenza virus by ClO₂ is due to oxidation and elimination of the function of the HA molecule.

RESULTS

Inhibition of haemagglutination by ClO₂

The ability of influenza virus particles to agglutinate chicken erythrocytes decreased when virus particles were treated with ClO₂ (Fig. 1a) with an IC₅₀ of 13.7 μM at 25 °C for 2 min and a half-life time for HA of 19.5 s at 25 °C with 100 μM ClO₂ (Fig. 1b). This inactivation process was temperature dependent (Fig. 1c), suggesting that the basis of the inhibition involved a chemical reaction. The activation energy of this inactivation process was determined by an Arrhenius plot (Fig. 1d). From the fitted line y = −1970x + 9.31 (r²=0.93), the activation energy was found to be 16.4 kJ·mol⁻¹·K⁻¹. Erythrocytes pre-treated with bacterial NA did not show agglutination (Fig. 1a), indicating that the agglutination occurred via sialic acid residues in the glycoproteins of the erythrocytes.
Inhibition of receptor binding of HA by ClO$_2$

The ClO$_2$-mediated inactivation of the ability of HA to bind its specific receptor was examined by direct binding of sialyl-$\alpha$(2,6)-lactose-$N$-acetylated $p$-phenylenediamine-derivatized human serum albumin conjugate (hereafter referred to as sialyl–albumin conjugate) to a microtitre plate pre-coated with HA. The ability of the conjugate to bind HA on the plate decreased markedly when HA was treated with ClO$_2$ (Fig. S1, available in JGV Online). Taken together, these results suggested that ClO$_2$ abolishes the ability of HA to bind to its specific receptor.

Modification of HA by ClO$_2$ as revealed by mass spectrometry (MS)

The precise nature of the ClO$_2$-induced modification of HA was investigated next. A tryptic digest of ClO$_2$-treated virus particles was performed, and the resulting peptides were separated by HPLC and the fractions subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. A fraction with a retention time of 25.0–25.5 min (Fig. S2, fraction 45) showed a mass-to-charge ratio ($m/z$) value of 976.55 (Fig. 2a). The tryptic fragment with $m/z$=976.55 corresponded to a peptide comprising 150NLLWLTGK157 of HA (H3 numbering), containing a single tryptophan, W153. However, the $m/z$ value of 976.55 was 32 mass units greater than that anticipated based on the amino acid sequence. Moreover, this peak was absent from virus particles that were not treated with ClO$_2$ (data not shown). The amino acid sequence of the peptide deduced from tandem MS (MS/MS) was 150NLLWLTGK157, except that W153 was 32 mass units larger than expected (Fig. 2b). This residue was tentatively assigned as $N$-formylkynurenine (FK) (Fig. 2a, inset) based on previous reports (Ogata, 2007; Stewart et al., 2008). The other MS peaks of the HPLC fractions are shown in Table S1.
Modification of synthetic peptide with ClO₂

A synthetic peptide of sequence NLLWLTGK treated with ClO₂ showed a novel peak with a retention time of 26.3 min, hereafter referred to as the 'ClO₂-modified peptide' (data not shown). As a control, another peptide, NLLGLTGK, with a substitution of G for W (bold), was also treated with ClO₂. This G-substituted peptide did not show any detectable change in mass after ClO₂ treatment (data not shown). These results suggested that the ClO₂-induced modification was caused by the presence of a tryptophan residue in peptide NLLWLTGK. To further verify this interpretation, the absorbance and fluorescence emission spectra of the ClO₂-modified peptide were compared with those of the original NLLWLTGK peptide. The absorbance and fluorescence spectral changes of the ClO₂-modified peptide indicated that its tryptophan residue was covalently modified (data not shown).

MS analysis of the ClO₂-modified peptide

The MALDI-TOF MS peak of the ClO₂-modified peptide (m/z=976.79) (Fig. S3a) was almost identical to the peak liberated after tryptic digestion from the ClO₂-treated virus particles (Fig. 2a). The spectrum obtained by MS/MS using the m/z=976.79 peak as a precursor ion also gave almost identical peaks (Fig. S3b) to those generated from the HA fragment released from the ClO₂-treated virus particles after trypsin digestion (Fig. 2b). Moreover, the amino acid composition of the ClO₂-modified peptide showed no tryptophan, although kynurenine was detected (data not shown). (Note that the formyl moiety of FK is removed under the acid hydrolysis conditions used for the amino acid analysis.) These results indicated that the synthetic peptide NLLWLTGK was also oxidized to NLL[FK]LTGK by ClO₂.

DISCUSSION

ClO₂ reacts non-specifically with tryptophan and tyrosine residues in proteins (Napolitano et al., 2005; Ogata, 2007; Stewart et al., 2008). However, it should be noted that ClO₂ does not necessarily react with all of the tryptophan and tyrosine residues in a specific protein (Ogata, 2007). The different reactivity of ClO₂ against amino acid residues in...
any given protein might depend on whether these residues are easily accessible to ClO₂ or not. This study demonstrated that ClO₂ reacts with W153 preferentially, if not specifically, in HA (Fig. 2). W153 is located on the surface of HA (Fig. 3a) and is presumably easily accessible to ClO₂. It is also possible that the geometry of W153 in HA facilitates the reaction with ClO₂. The binding ability of HA to its specific receptor on a target cell is crucial for establishing infection (Skehel & Wiley, 2000). Because W153 in HA is highly conserved in almost all subtypes of influenza virus (Knossow & Skehel, 2006; Martín et al., 1998), it is believed to play a crucially important role in the function of HA (Meisner et al., 2008). As shown in Fig. 3(a, b), W153 is located at the bottom of the receptor-binding site just below the sialic acid residue of the receptor (Lin et al., 2009). In fact, the acetamide moiety (Fig. 3b) of the sialic acid is in van der Waals contact with the indole ring of W153 (Lin et al., 2009; Martin et al., 1998). It is speculated that W153 together with Y195, which makes a hydrogen bond with the pyrrole nitrogen of W153, provides an optimal geometric structure that allows stable binding of the sialic acid residue of the receptor to HA. This proposed interaction explains why oxidation of W153 by ClO₂ is likely to abolish the function of HA (Fig. 1).

It was shown previously by isotopic labelling experiments that ClO₂ oxidizes tryptophan to FK by transferring two atoms of oxygen from ClO₂ (Ogata, 2007). In this reaction, the pyrrole ring of tryptophan is split, and the benzene ring of W153 will shift as shown in Fig. 3(c, lower arrow). In addition, because the N-formyl bond in FK is a freely rotatable σ bond, the oxygen atom of the formyl group may rotate upwards (in the direction of the sialic acid residue; Fig. 3c, upper arrow) to accommodate the bulky CO moiety. These possible conformational changes in the binding site of HA are likely to perturb its interaction with the sialic acid residue. Additionally, such a conformational change in the receptor-binding site of HA would no longer enable formation of the necessary hydrogen bonds involving Y95, Y195, E190 and Q226 (Fig. 3a, dotted lines) to hold the sialic acid residue in place (Sauter et al., 1992). The structural alterations to the binding site caused by the oxidized W153 would therefore hinder the function of the HA molecule.

**METHODS**

**Virus and reagents.** Influenza A virus [A/New Caledonia/20/99 (H1N1)] was obtained from Dr Y. Okuno of the Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, Japan. Antibodies were purchased from MyBioSource. Peptides (>98% pure) were purchased from Pi Proteomics. Recombinant HA protein of A/ New Caledonia/20/99 (H1N1) was purchased from Protein Sciences. ClO₂ was prepared using NaClO₂ and HCl as described previously (Ogata, 2007). The sialyl–albumin conjugate was obtained from IsoSep AB (Kallin et al., 1986). This conjugate has 15 moles of the oligosaccharide moiety per mole of the protein. Trypsin was obtained from Promega. All other reagents were analytical grade and were obtained from Nacalai Tesque. Influenza virus particles were prepared in embryonated chicken eggs based on the method described by Herrmann (1978). Approximately 3.5 mg virus protein in 1 ml was obtained from 100 eggs using this method.

**Treatment of virus particles and HA with ClO₂.** Unless otherwise specified, the standard reaction for the treatment of virus particles or HA protein with ClO₂ was carried out in a 1 ml reaction mixture containing 10 mM sodium phosphate buffer (pH 7.0), 130 mM NaCl, 300 µM ClO₂ and virus particles (100 µg protein ml⁻¹) or HA protein (100 µg ml⁻¹). The reaction was started by the addition of ClO₂ and was continued at 25 °C for 2 min. The reaction was terminated by the addition of a twofold molar excess of Na₂S₂O₃ (Ogata, 2007). For a
control experiment without ClO₂ treatment, ClO₂ that had been pre-
mixed with a twofold molar excess of Na₂SO₃ was added to the
reaction mixture. For the reaction with virus particles, the reaction
mixture was next heated at 100 °C for 3 min, followed by the addition
of 38 μl trypsin solution (1 mg ml⁻¹). The mixture was incubated at
37 °C for 5 h and then centrifuged at 20000 g at 4 °C for 1 h. The
supernatant was recovered and a 100 μl aliquot was subjected to HPLC.

Haemagglutination assay. A round-bottomed, 96-well polystyrene
microwell plate (Biotec) was used to measure the haemagglutination
ability of virus particles. Virus particles that had been treated or not
with ClO₂ were suspended in PBS. The virus particles were then
diluted by twofold serial dilutions and placed in each well of the plate
(50 μl per well). Fresh chicken erythrocytes, treated or not with
Clostridium perfringens NA (0.39 U ml⁻¹, 37 °C for 10 min in PBS),
were suspended in PBS at a concentration of 4 × 10⁷ cells ml⁻¹ and
then added to each well (50 μl per well). The plate was kept at 4 °C
for 1 h. Agglutination of erythrocytes was observed by eye: a sheet of
erthrocytes was formed on the bottom of the well if agglutination
occurred; otherwise the erythrocytes formed a red ‘spot’ on the bottom
in the centre of the well. One haemagglutinin unit was defined as the
minimum amount of virus that agglutinated erythrocytes under the
assay conditions described above.

Receptor-binding assay of HA. Recombinant HA, treated or not
with ClO₂ as described above, was diluted with PBS to a
concentration of 20 μg ml⁻¹. One hundred microlitres of this
solution was then placed in each well of a 96-well, flat-bottomed
polystyrene microwell plate (MaxiSorp; Nunc) at 25 °C for 30 min.
Each well of the plate was washed three times with 100 μl PBS
containing 0.01 % (v/v) Tween 20 and once with 100 μl distilled
water. Equine skeletal muscle myoglobin (Sigma-Aldrich) solution in
PBS (1 mg ml⁻¹) was placed in each well (100 μl per well) at 25 °C
for 5 min to block the protein-uncoated area of the well. The wells
were washed as described above, air dried and stored at room
temperature, protected from light, until use. For the receptor-binding
assay, 100 μl sialyl-albumin (1.5 μg ml⁻¹), as a model of truncated
HA receptor, was placed in each well at 25 °C for 5 min. The wells
were then washed as described above, and 100 μl HRP-labelled anti-
human serum albumin antibody (diluted to 0.8 μg ml⁻¹) was placed
in each well at 25 °C for 5 min. The wells were then rinsed as described
above before finally adding peroxidase substrate solution
(Bio-Rad). The reaction was allowed to continue at 37 °C for 10 min
before quenching by the addition of 20 mM HCl (100 μl per well).
The plate was scanned using a microplate reader (model MPR-A4;
Toyosoda) to measure the absorbance at 415 nm. The ability of the
peptides, as described previously (Ogata, 2007), to bind HA was
measured in a separate experiment.

HPLC of peptides. Peptides (5–20 nmol in 100 μl) were resolved on
a Cosmosil 5C₁₈-AR-300 column (4.6 × 250 mm; Nacalai Tesque)
mounted on an HPLC system. After injection of a peptide sample, the
column was eluted with 0.1 % (v/v) trifluoroacetic acid containing
two consecutive linear gradients of acetonitrile (0–12 %, v/v, from 0
to 1 min, and then 12–60 %, v/v, from 1 to 60 min) using a flow rate
of 1 ml min⁻¹. An elution profile of the peptides was obtained by
monitoring the absorbance of the eluate at 230 or 280 nm. Fractions of
the eluate were lyophilized and dissolved in distilled water for
further analysis.

MS of peptides. Peptide samples (10–500 pmol) were subjected to
either MALDI-TOF MS to measure their intact mass, which is actually the
m/z ratio (Biemann, 1988), or MS/MS, after electron

spray ionization of precursor ions, to analyse the amino acid sequences
of the peptides, as described previously (Ogata, 2007).

Amino acid analysis of peptides. The amino acid composition of the
peptides (~200 pmol) was analysed using an amino acid analyser
(model L-8500; Hitachi) after acid hydrolysis (in 6 M HCl at 110 °C
for 22 h) under reduced pressure and in the presence of nitrogen.
Thioglycolic acid (4 %, v/v) was added to the HCl to prevent
decomposition of tryptophan during hydrolysis.

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