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Cu^{2+}-catalyzed Oxidative Degradation of Thyroglobulin

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Thyroglobulin (Tg) was subjected to metal-catalyzed oxidation, and the oxidative degradation was analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. In contrast to no effect of hydrogen peroxide (H_2O_2) alone on the Tg degradation, the inclusion of Cu^{2+} (30 μM), in combination with 2 mM H_2O_2, caused a remarkable degradation of Tg, time- and concentration-dependent. The action of Cu^{2+} was not mimicked by Fe^{2+}, suggesting that Tg may interact selectively with Cu^{2+}. A similar degradation of Tg was also observed with Cu^{2+}/ascorbate system, and the concentration of Cu^{2+} (5–10 μM), in combination with ascorbate, required for the effective degradation was smaller than that of Cu^{2+} (10–30 μM) in combination with H_2O_2. In support of involvement of H_2O_2 in the Cu^{2+}/ascorbate action, catalase expressed a complete protection. However, hydroxyl radical scavengers such as dimethylsulfoxide or mannitol failed to prevent the oxidation of Tg whereas phenolic compounds, which can interact with Cu^{2+}, diminished the oxidative degradation, presumably consistent with the mechanism for Cu^{2+}-catalyzed oxidation of protein. Moreover, the amount of carbonyl groups in Tg was increased as the concentration (3–100 μM) of Cu^{2+} was enhanced, while the formation of acid-soluble peptides was not remarkable in the presence of Cu^{2+} up to 200 μM. In further studies, Tg pretreated with heat or trichloroacetic acid seemed to be somewhat resistant to Cu^{2+}-catalyzed oxidation, implying a possible involvement of protein conformation in the susceptibility to the oxidation. Based on these observations, it is proposed that Tg could be degraded non-enzymatically by Cu^{2+}-catalyzed oxidation.

Keywords: Thyroglobulin, copper ions, hydrogen peroxide, ascorbic acid, oxidative degradation

INTRODUCTION

Thyroglobulin (Tg), present as a homodimer (2 × 330 kDa) in the thyroid gland, has the ability to form 3,3′,5-triiodothyronine or thyroxine residues through the coupling of two iodotyrosine residues.[1] The oxidative coupling, which depends on the native structure of Tg, is catalyzed by thyroid peroxidase in the presence of a hydrogen peroxide (H_2O_2)-generating system.[2] In thyroid tissue, the H_2O_2-generation involves NADPH oxidase, which is responsible for the formation of $O_2^{-\bullet}$. In turn, $O_2^{-\bullet}$ is transformed to H_2O_2 in the presence of superoxide dismutase.[3] During the enzymatic peroxidative reaction,

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thyroid peroxidase is oxidized to two forms, porphyrin π-cation radical and protein radical as intermediates.[4,5] Meanwhile, H_2O_2 can be converted to $^{\bullet}OH$ non-enzymatically.[6] Thus, various reactive oxygen radicals could be generated during oxidative reactions in the thyroid tissue.

Previously, Tg molecules had been observed to form dimeric molecules via intermolecular cross-linking during oxidative iodination.[7] The cross-linking, resulting in the dimerization of Tg molecules, was suggested to be ascribed to the 3-3′-dityrosine bridges in the Tg molecule. Conversely, a recent study[8,9] showed that the mild oxidation of multimeric Tg form using thyroid peroxidase and a H_2O_2-generating system resulted in the gradual degradation of Tg. Thus, it was supposed that reactive oxygen species could be involved in multimerization of Tg as well as its degradation.

It is well established[10] that the exposure of proteins to reactive oxygen species can cause the alteration of the primary structure, followed by gross distortion of secondary and tertiary structure; proteins exposed to $^{\bullet}OH$ undergo progressive covalent cross-linking as well as molecular degradation. Under aerobic conditions, $^{\bullet}OH$-catalyzed degradation of proteins, accompanied by the formation of new carbonyl groups, is more favorable. In this respect, metal-catalyzed oxidation employing iron or copper ions, which are known to generate potent oxidant radicals such as $^{\bullet}OH$ via a Fenton-type reaction,[6] might cause the structural alteration of Tg under aerobic conditions. Moreover, the accumulation of transition metal ions in the thyroid tissue could promote the formation of reactive oxygen molecules such as $O_2^{-\bullet}$ or $^{\bullet}OH$ in this tissue. In addition, ascorbic acid can be a contributory factor, since the reducing agent also plays a role as a prooxidant.[11] Despite this possibility, metal-catalyzed oxidation of Tg was not extensively examined, except the report [12] that Tg was utilized as one of the non-lenticular proteins, which were non-specifically oxidized by a drastic oxidation condition employing 100 μM Cu^{2+} and 50 mM H_2O_2.

Whereas most of the studies concerning the regulation of thyroid hormone biosynthesis have emphasized the modulation of thyroid peroxidase activity, responsible for the oxidative iodination of tyrosine residues,[13–15] the investigation of the structural change of Tg was limited to some reports,[7,16,17] concerned with the posttranslational modifications or carbohydrate degradation. Since oxidized proteins are more susceptible to subsequent proteolytic degradation, the metal-catalyzed oxidation of Tg might facilitate the proteolytic degradation of Tg,[6,11] finally resulting in the release of more thyronine molecules.

Here, we examined the metal-catalyzed oxidative degradation of Tg, and report that Tg is one of the proteins sensitive to Cu^{2+}-catalyzed oxidation.

MATERIALS AND METHODS

Chemicals and Reagents

Porcine Tg (iodine content, approximately 1%), mannitol, glutathione, dimethylsulfoxide, diiodotyrosine, sodium dodecylsulfate (SDS), acrylamide, ascorbic acid, quercetin, catalase (bovine liver, 1880 U/mg), and Sephacryl S-400-HR were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2,4-Dinitrophenylhydrazine and H_2O_2 (30%) were provided by Junsei Chemical Co. (Tokyo, Japan). Metal ions including cupric sulfate and ferrous sulfate were of analytical grade. Human goitre Tg was provided kindly by Dr. B. Mallet (Unite 38 INSERM, France).

Exposure of Tg to Oxidative System

Tg (0.8 mg/ml) was preincubated with H_2O_2 (2 mM), transition metal (30 μM) or the combination of H_2O_2 and transition metal in 50 mM HEPES buffer, pH 7.4 (30 μl) at 38°C for 10 min, and an aliquot (6 μl) was taken and subjected to SDS-polyacrylamide gel electrophoresis[18] denaturing under reducing condition to see the degradation of Tg.

Oxidative Degradation of Tg by Cu^{2+}/H_2O_2 or Fe^{2+}/H_2O_2 System

Tg was exposed to 30 μM Cu^{2+} or Fe^{2+} in the presence of H_2O_2 at various concentrations (0.3–2 mM) at 38°C in 50 mM HEPES buffer, pH 7.4 (30 μl) for 10 min, and then an aliquot (6 μl) was taken for the SDS-PAGE analysis. Next, Tg was exposed to 2 mM H_2O_2 in the presence of Cu^{2+} at various concentrations (3–100 μM). Separately, Tg was exposed to the combination of Cu^{2+} (30 μM) and H_2O_2 (2 mM) for various times (0–10 min).

Oxidative Degradation of Tg by Cu^{2+}/Ascorbate System

Tg (0.8 mg/ml) was exposed to 2 mM ascorbic acid in the presence of Cu^{2+} at various concentrations (2–30 μM) in 50 mM HEPES buffer (30 μl), pH 7.4 at 38°C for 10 min, and an aliquot (6 μl) was taken and subjected to SDS-PAGE analysis. Separately, Tg was exposed to 1 mM ascorbic acid in the presence of Cu^{2+} at various concentration (1–10 μM) at 38°C for 2 h.

Oxidative Degradation of Heat-, Acid- or Detergent-treated Tg by Cu^{2+}/Ascorbate System

Tg (0.8 mg/ml) was pretreated in different conditions prior to the exposure (10 min) to the combination of Cu^{2+} (30 μM) and ascorbic acid (1 mM). Heat pretreatment was carried out by boiling Tg (0.8 mg/ml) in 50 mM HEPES buffer, pH 7.4 for 20 min, and SDS pretreatment was done by storing Tg in 1.5% SDS at room temperature. Separately, Tg was pretreated with trichloroacetic acid (5%), and after neutralization, the pellet was sonicated.

Prevention against Oxidative Degradation of Tg by Cu^{2+}/Ascorbate System

Cu^{2+} (30 μM)/ascorbate (1 mM)-mediated oxidation of Tg was performed in the presence or absence of catalase or each compound, and 10 min later an aliquot (6 μl) was taken for the SDS-PAGE analysis. Separately Cu^{2+}/ascorbate-induced oxidation was performed in the presence of HEPES (10–200 mM) or DMSO (50–450 mM) of different concentrations.

Determination of Carbonyl Group in Tg Exposed to Cu^{2+}/H_2O_2 System

Tg (0.8 mg/ml) was exposed to Cu^{2+} (3–100μM) and H_2O_2 (2 mM) in 300 μl of 50 mM HEPES buffer (pH 7.4) at 38°C for 10 min, and to the mixture was added 600 μl of 20% trichloroacetic acid. After centrifugation (3000*g*, 10 min), to the pellet portion was added 1 ml of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl, and the mixture was allowed to stand at 38°C for 50 min under intermittent vortexing. Trichloroacetic acid treatment and centrifugation were repeated, and the pellet was washed with 1 ml of ethanol/ethyl acetate solution (1 : 1) to remove free reagent as reported previously.[19] Finally, the protein pellet was dissolved in 1 ml of 6 M guanidine solution in 20 mM phosphate buffer (pH 2.3), and the absorbance of the clear solution was measured at 380 nm ($\varepsilon_{max} = 22\,000$).

Sephacryl S 400 HR Gel Chromatography

Tg (2.4 mg/ml) was exposed to Cu^{2+} (5 μM) and ascorbic acid (2 mM) in 30 μl of 50 mM HEPES buffer (pH 7.4) at 38°C for 10 min, and 4 volumes of the reaction mixture (120 μl) were loaded onto a sephacryl S 400 HR gel column (1.7 × 25 cm), which was eluted with 20 mM Tris buffer (pH 7.0) containing 0.5 M NaCl at a flow rate of approximately 18 ml/h. Tg, which was not subjected to oxidative condition, was used as control.

Polyacrylamide Gel Electrophoresis

Each sample (6 μl) of Tg, boiled in 125 mM Tris buffer, pH 6.8 containing 1.6% SDS and 80 mM mercaptoethanol, was subjected to SDS-PAGE

under reducing and denaturing conditions as described previously.[18] Stacking gel and running gel were 4% and 6%, respectively.

RESULTS

In an attempt to examine oxidant-induced degradation of Tg, Tg (0.8 mg/ml) was treated with H_2O_2, transition metal ions or the combination of H_2O_2 and metal ions, and its molecular behavior was determined by PAGE analyses under reducing and denaturing conditions. Since there was no remarkable change in the behavior of Tg molecule after the exposure to H_2O_2, Fe^{2+} or Cu^{2+} alone (data not shown), the effect of the combination of H_2O_2 and divalent metal ions such as Fe^{2+} or Cu^{2+} was examined. In contrast, Tg was extensively cleaved after exposure to Cu^{2+}/H_2O_2 system as evidenced by disappearance of Tg band in the PAGE analysis (Figure 1A). In further studies, where the concentration of Cu^{2+} or Fe^{2+} was fixed at 30 μM, and that of H_2O_2 was varied, it was found that the cleavage of Tg molecules by Cu^{2+}/H_2O_2 system was dependent on the concentration of H_2O_2; a slight degradation was found in the combination with 0.3 mM H_2O_2 and the combination with 2 mM H_2O_2 expressed an almost complete degradation. In contrast, H_2O_2 up to 2 mM, in combination with Fe^{2+} (30 μM), failed to exhibit a significant degradation of Tg. When the concentration of H_2O_2 was fixed at 2 mM, and that of Cu^{2+} was varied (Figure 1B), a concentration-dependent effect of Cu^{2+} was found; a partial degradation of Tg was observed in the combination with 10 μM Cu^{2+} and a complete one in the combination with 30 μM Cu^{2+}. Time-dependency (Figure 1C) indicates that the radical-mediated degradation of Tg started 2 min after the exposure of Tg to Cu^{2+}/H_2O_2 system, and was also complete after 10 min. No aggregated forms of Tg were visible after the exposure to Cu^{2+}/H_2O_2 system. Separately, when Tg of various concentrations (0.8–3.2 mg/ml) was exposed to Cu^{2+}/H_2O_2 system and the degradation

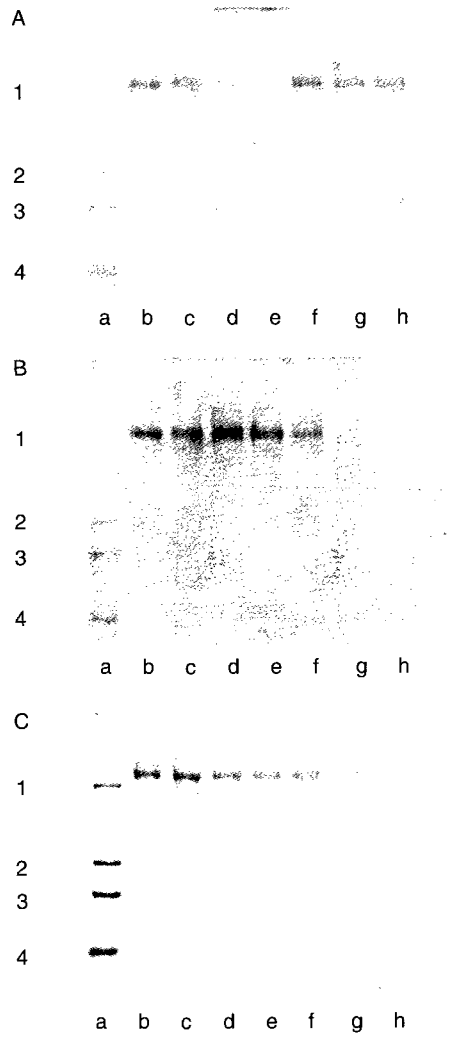

FIGURE 1 Oxidative degradation of Tg Cu^{2+}/H_2O_2 or Fe^{2+}/H_2O_2 system. Porcine Tg (0.8 mg/ml) was incubated with H_2O_2 and transition metal in 50 mM HEPES buffer, pH 7.4 at 38°C and 10 min later, an aliquot (6 μl) was taken for SDS-PAGE analysis employing 6% acrylamide gel. A. The concentration of H_2O_2 was varied (0.3–2 mM), while that of Cu^{2+} or Fe^{2+} was fixed at 30 μM. Lane a, molecular weight markers (M.W; 205 kDa, 116 kDa, 97.4 kDa, 66 kDa; markers 1, 2, 3 and 4, respectively); lane b, Tg only; lanes c–e, $+Cu^{2+}$ and H_2O_2 (0.3, 1.0 and 2.0 mM, respectively); lanes f–h, Fe^{2+} and H_2O_2 (0.3, 1.0 and 2.0 mM, respectively). B. The concentration of Cu^{2+} was varied (3–100 μM), while that of H_2O_2 was fixed at 2 mM. Lane a, markers; lane b, Tg only; lane c, $+Cu^{2+}$ (100 μM); lane d, $+H_2O_2$ (2 mM), lanes e–h, $+H_2O_2$ and Cu^{2+} (3, 10, 30 and 100 μM, respectively). C. Tg (0.8 mg/ml) was incubated at 38°C with H_2O_2 (2 mM) and Cu^{2+} (30 μM) for various times (0–10 min). Lane a, markers; lane b, Tg only (10 min incubation without oxidation); lane c, Tg only (not incubated); lanes d–h, $+Cu^{2+}$ and H_2O_2 (0, 1, 2, 5 and 10 min incubation, respectively).

of Tg was analyzed, the degradation of Tg was dependent on the concentration of Tg used (data not shown); 30 μM Cu^{2+}/2 mM H_2O_2 was capable of completely degrading Tg at 0.8 mg/ml, but not Tg at 1.6 mg/ml or higher concentrations, indicating that Cu^{2+}-catalyzed oxidation requires a limited molar ratio of Tg to Cu^{2+} for effective cleavage. In related experiments (data not shown), Cu^{2+}-catalyzed oxidative cleavage of Tg molecule was analyzed by the quantitative measurement of acid-soluble material; there was no obvious formation of trichloroacetic acid-soluble peptides under the routine oxidation condition where 30 μM Cu^{2+} was combined with 2 mM H_2O_2. Meanwhile, the increase of H_2O_2 concentration to 5 and 10 mM resulted in the formation of soluble peptides, corresponding to approximately 0.12% and 0.3% cleavage of total Tg, respectively.

The formation of carbonyl groups is one of the characteristics of metal-catalyzed oxidation of proteins. First, the amount of carbonyl content in porcine Tg or human goiter Tg was determined. The amount of carbonyl group in non-treated Tg was estimated to be approximately 0.19 nmol per nmole of Tg, in contrast to 0.073 nmol per nmole of goitre Tg. When Tg was exposed to Cu^{2+}/H_2O_2 system, it was revealed (Figure 2) that the amount of carbonyl group was enhanced from 0.21 nmol per nmole of Tg to 5.04 nmol per nmole of Tg as the concentration of Cu^{2+}, in combination with 2 mM H_2O_2, was augmented from 3 to 100 μM. Thus, the response of Tg to the oxidative action of Cu^{2+}/H_2O_2 system was reaffirmed by the formation of carbonyl groups in the oxidation product of Tg. In contrast, the incubation with 2 mM H_2O_2 in the presence of Fe^{2+} up to 30 μM caused no remarkable increase (< 0.2 nmol per nmole Tg) of carbonyl groups.

After establishing the Cu^{2+}-catalyzed oxidative degradation of Tg, we turned to the oxidation of Tg by the combination of Cu^{2+} and ascorbic acid or glutathione as a prooxidant. The Cu^{2+}/glutathione system failed to cause a degradation of Tg. When Tg was exposed to ascorbic acid in the presence of Cu^{2+} at various concentrations (2–30 μM), it was found that a remarkable degradation was exhibited by ascorbic acid (2 mM) in combination with Cu^{2+} at concentrations as low as 10 μM (data not shown). When the incubation time was extended over 2 h (Figure 3), a partial degradation was expressed by 1 mM ascorbic acid in combination with 2.5 μM Cu^{2+}, and an almost complete cleavage was shown by the combination with Cu^{2+} at 5 μM or higher concentrations, indicating that the concentration of Cu^{2+}, in combination with ascorbate, required for the effective oxidative degradation was lowered.

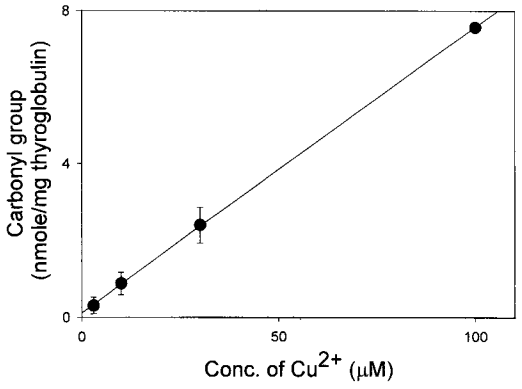

FIGURE 2 Determination of carbonyl group in Tg exposed to Cu^{2+}/H_2O_2 system. Tg (0.8 mg/ml) was incubated at 38°C with H_2O_2 (2 mM) and Cu^{2+} (3–100 μM) for 10 min. The amount (nmol/mg Tg) of carbonyl group in Tg oxidized was determined as described in Methods. Values are presented as mean ± SD of three experimental sets.

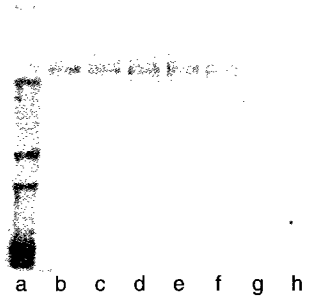

FIGURE 3 Oxidative degradation of Tg by Cu^{2+}/ascorbate system. Tg (0.8 mg/ml) was exposed to 1 mM ascorbic acid in the presence of Cu^{2+} at various concentrations (1–10 μM) at 38°C for 2 h. Lane a, markers; lane b, Tg only; lane c, +Cu^{2+} (10 μM); lane d, +ascorbic acid (1 mM); lanes e–h, +ascorbic acid (1 mM) and Cu^{2+} (1, 2.5, 5 and 10 μM, respectively).

Thus, it appeared that the Cu^{2+}/ascorbate system was found to be more effective than Cu^{2+}/H_2O_2 system for the oxidative degradation of Tg. In further studies (data not shown), it was found that compared to the Cu^{2+} (30 μM)/H_2O_2 (2 mM) system, the Cu^{2+} (30 μM)/ascorbic acid (1 mM) system was more effective to degrade Tg at a higher concentration (1.6 mg/ml). Separately, the Cu^{2+}/ascorbate-mediated oxidation of Tg molecule was examined by Sephacryl gel filtration chromatography. Figure 4 demonstrates that Tg fragments of smaller size were produced as major degradation product from the exposure of Tg to Cu^{2+}/ascorbic acid, in contrast to a trace amount of aggregated forms of higher molecular weights.

To prove an intermediary role of H_2O_2 in the oxidative degradation of Tg by the Cu^{2+}/ascorbate system, Tg was exposed to Cu^{2+}/ascorbate in the presence of catalase. As demonstrated in Figure 5, catalase (1,650 U/ml) exerted a remarkable protection against Cu^{2+}-catalyzed degradation of Tg, indicative of the involvement of H_2O_2 in Cu^{2+}-catalyzed oxidative degradation. Meanwhile, heat-treated catalase failed to prevent the oxidative degradation of Tg (data not shown).

Next, in an attempt to elucidate the mechanism by which Tg is susceptible to the Cu^{2+}-catalyzed oxidative inactivation, Tg was exposed to the Cu^{2+}/ascorbate system in the presence of general $^{\bullet}OH$ scavengers. Neither mannitol (100 mM) nor DMSO (50–450 mM) prevented the oxidative cleavage, excluding the possibility that the oxidative degradation of Tg might be due to the freely diffusible $^{\bullet}OH$ radicals. Separately, Cu^{2+}/ascorbate-induced oxidation was carried out in HEPES buffer (pH 7.4) which is able to scavenge OH radicals[20] at different concentrations (10, 50 and 200 mM), However, there was no remarkable difference of oxidative degradation between various concentrations of HEPES buffer. Meanwhile, quercetin (1 mM) and diiodotyrosine (1 mM), which are able to interact with copper ions,[21,22] expressed a substantial prevention against Cu^{2+}/ascorbate-mediated cleavage of Tg. In further studies (data not shown), glutathione (100 μM) was found to be effective in preventing against Cu^{2+}/ascorbate-mediated oxidative cleavage of Tg.

In order to see if the oxidative cleavage of Tg is related to the structure of Tg, Tg was pretreated with 20 min boiling, 5% trichloroacetic acid or

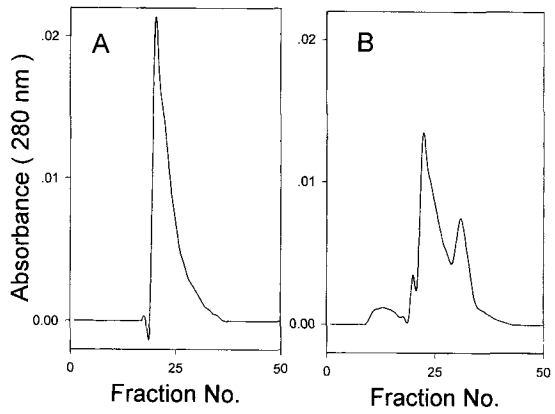

FIGURE 4 Gel filtration chromatography of Tg exposed to Cu^{2+}/ascorbate system. Tg (2.4 mg/ml) was exposed to the combination of Cu^{2+} (50 μM) and ascorbate (2 mM) in 30 μl of 50 mM Tris buffer at 38°C for 20 min, and the pooled sample (120 μl) of four sets was loaded onto the Sephacryl S-400 HR gel column which was eluted with 20 mM Tris buffer, pH 7.0 containing 0.5 M NaCl (flow rate, approximately 18 ml/h). A, control; B, oxidized Tg.

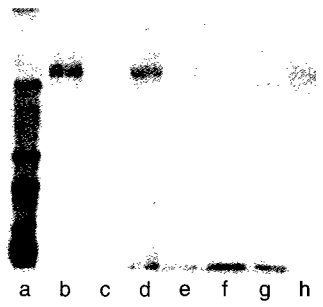

FIGURE 5 Prevention against oxidative degradation of Tg by Cu^{2+}/ascorbate system. Tg was exposed to Cu^{2+} (30 μM) and ascorbic acid (1 mM) in 50 mM HEPES buffer, pH 7.4 containing catalase or each compound as described in Figure 5. Lane a, markers; lane b, Tg only; lane c, Tg oxidized with Cu^{2+} and ascorbic acid; lane d, oxidation +catalase (1650 U/ml); lane e, oxidation +dimethylsulfoxide (50 mM); lane f, oxidation +mannitol (100 mM); lane g, oxidation +diiodotyrosine (1 mM); lane h, oxidation +quercetin (1 mM).

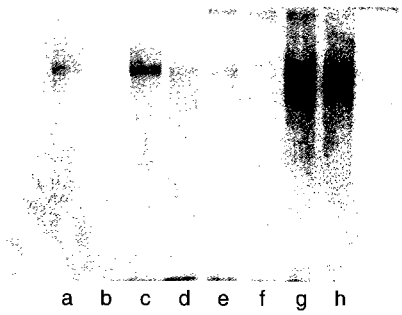

FIGURE 6 Oxidative degradation of heat-, acid- or detergent-treated Tg by Cu^{2+}/ascorbate system. Tg (0.8 mg/ml), pretreated in different condition, was exposed to the combination of Cu^{2+} (30 μM) and ascorbic acid (1 mM). Lane a, Tg only; lane b, oxidized with Cu^{2+} and ascorbic acid; lane c, pretreated with SDS; lane d, pretreated with SDS and oxidized; lane e, pretreated with 20 min boiling; lane f, pretreated with boiling and oxidized; lane g, pretreated with trichloroacetic acid; lane h, pretreated with trichloroacetic acid and oxidized.

1.5% SDS, and then subjected to the Cu^{2+}/ascorbate-mediated oxidation. Figure 6 demonstrates that all of treated Tgs except the boiled protein seemed to be, although partially, resistant to the Cu^{2+}/ascorbate system. The greatest resistance was observed with trichloroacetic acid treatment. Hence it is supposed that the susceptibility of Tg to oxidant radicals may reside, at least in part, in the protein conformation. The heat treatment, prior to the oxidation with copper ions and ascorbate, appeared to generate some aggregated protein, which was not dissociated under reducing conditions.

DISCUSSION

The cleavage of some proteins and the production of aggregates can be achieved using radical-generating systems. Hydroxyl radical attack on some proteins causes extensive O_2-dependent fragmentation while cross-linking is observed predominantly in the absence of O_2.[10] Reactive oxygen species are considered deleterious in part because accumulation and degradation of oxidized proteins are associated with various diseases.[5] Conversely, reactive oxygen species may show a beneficial role in the fate of multimeric Tg; the oxidative fragmentation of insoluble multimeric Tg leads to the formation of soluble Tg fragments.[8,9]

Earlier, it had been suggested that the intermolecular tyrosine cross-links, resulting in the formation of dityrosine residues, in addition to the formation of disulfide bridges occurred during the oxidative iodination of Tg by thyroid peroxidase.[7] This intermolecular cross-linking of Tg leads to the oligomerization of Tg molecules. Meanwhile, the mild oxidation employing thyroid peroxidase and H_2O_2 was found to convert the insoluble multimerized Tg, which bears disulfide and dityrosine bridges, to soluble forms of Tg. Interestingly, the enzymatic reduction of disulfide bridge in multimeric Tg was important for the generation of soluble Tg molecules which were able to synthesize T_4 residues.[9] Thus, it was suggested that the peroxidative degradation of Tg molecule could affect the fate of role of Tg.

In this respect, metal-catalyzed oxidation of Tg could exert a dual action; it can catalyze the oxidative cross-link of cysteine residues or tyrosine residues whereas it can mediate the decomposition of Tg molecule at a specific site. However, the former possibility is less likely for highly-iodinated Tgs such as porcine Tg, which already underwent the oxidative process, as suggested from no remarkable formation of multimeric molecules after the exposure of porcine Tg to Cu^{2+}/H_2O_2 system under our conditions. Moreover, there was no remarkable difference in the Cu^{2+}-catalyzed oxidative degradation between goitre Tg, iodinated to a low level,[1] and porcine Tg, highly-iodinated (data not shown). Thus, it is not likely that the intermolecular dimerization through dityrosine linkage is involved principally in the oxidation of Tg by Cu^{2+}/H_2O_2. Meanwhile, the oxidative cleavage of Tg is well evidenced by the disappearance of Tg molecules in gel electrophoresis analyses. This is also supported by the result from gel filtration chromatography, although the pattern of cleavage in gel chromatography does not coincide with that in PAGE

analysis. This might be due to the different conditions of Tg treatment or migration. A further support may come from the formation of carbonyl groups, a common marker of metal-catalyzed oxidation, in Tg exposed to Cu^{2+}/H_2O_2 system.[6] Thus, the action of Cu^{2+}-catalyzed oxidation of Tg may follow the mechanism[10] established for the O_2-dependent hydroxyl radical attack of proteins, where the main oxidation process is the degradation rather than multimerization. The same result was also obtained from the incubation of Tg with the Cu^{2+}/ascorbate system. The concentration of Cu^{2+} required for the effective degradation of Tg was lower in combination with ascorbic acid than in combination with H_2O_2. In addition, the Cu^{2+}/ascorbate system cleaved a higher amount of Tg than Cu^{2+}/H_2O_2 system. The higher efficacy of Cu^{2+}/ascorbate system, compared to Cu^{2+}/H_2O_2 system, might be explained by the facilitated redox cycle of copper ion in the presence of ascorbic acid. The oxidant species responsible for the cleavage of Tg by Cu^{2+}/ascorbate may be derived from H_2O_2 as suggested from the protective effect of catalase against the oxidative cleavage of Tg by Cu^{2+}/ascorbate. The possible participation of $^{\bullet}OH$ in the oxidative degradation is well evidenced from the effective degradation of Tg molecules in the presence of Cu^{2+} and H_2O_2. However, the involvement of extraneous $^{\bullet}OH$ is excluded since $^{\bullet}OH$ scavengers[20] such as Hepes, mannitol or dimethyl sulfoxide failed to prevent the oxidative cleavage of Tg. Even high concentrations of DMSO (450 mM) or HEPES (200 mM) had no effect on the oxidative degradation. Instead, Cu^{2+}-bound $^{\bullet}OH$ at the specific site is supposed to be responsible for the oxidative cleavage, as reported with metal-catalyzed oxidation of proteins.[6] Presumably consistent with this, compounds which interact with Cu^{2+} expressed some protection against the oxidative cleavage of Tg. Earlier, pyrane ring-containing phenols such as quercetin had been observed to interact with Cu^{2+}.[21] The protective action of quercetin might be due to its ability of binding to Cu^{2+} as well as its antioxidant property. Also, diiodotyrosine,[22] which can interact with Cu^{2+}, showed a good protection. In addition, glutathione, a thiol, prevented the oxidative degradation of Tg. Although the binding site of Cu^{2+} was not characterized in this study, the relatively low concentration of Cu^{2+} required tor the degradation of Tg, taken with the metal selectivity, implies a potential existence of Cu^{2+}-binding sites in the Tg molecule. A support for this notion may come from the low molar ratio (8–24) of Cu^{2+}/Tg for the effective degradation. Interestingly, at these molar ratios, approximately 0.6–1.6 mol of carbonyl group was produced from 1 mol of Tg exposed to the Cu^{2+}-catalyzed oxidation. From these data, it is supposed that Cu^{2+}-catalyzed degradation may be related to the formation of carbonyl groups in Tg.

One possible explanation of the resistance of a high concentration of Tg to radical-induced cleavage could be that the Tg may actively scavenge reactive oxygen species via a peroxidative decomposition of hydrogen peroxide. A recent report[23] suggests that some amino acid residues such as cysteine or methionine might play an antioxidant role. Alternatively, the Cu^{2+} may be trapped by amino acid residues, such as cysteine, in the primary structure of Tg. Also, the conformation of Tg might be important for the interaction of Tg with Cu^{2+}, since the treatment with trichloroacetic acid, heat or SDS decreased the susceptibility of Tg to Cu^{2+}-catalyzed oxidation. However, since the effect of these treatments was not complete, it is possible that some part of the oxidative cleavage might be ascribed to the presence of randomly arranged appropriate amino acid residues, which can associate with copper ions. Moreover, the possibility that SDS could affect the generation of hydroxyl radicals during the incubation is not excluded. It is of note that boiling generates a dimer-like form of Tg, which was not reduced in PAGE analysis in the presence of mercaptoethanol. This might be in part explained by the assumption that some part of Tg may undergo non-reducible dimerization such as dityrosine bridge formation, which needs further studies. From these results, it is proposed that the

multimerization of Tg molecule through dityrosine or disulfide formation might contribute to the resistance of Tg to the Cu^{2+}-catalyzed oxidation.

An oxidative cleavage of Tg by Cu^{2+}/H_2O_2 system may reveal previously-shielded amino acid residues as newly-preferred proteolytic substrate. Such fragments that were liberated by the reaction of Tg with the oxidative degradation, may be more readily cleaved by intracellular proteases[24,25] to smaller peptides containing thyronine residues. Previously, it had been reported that thyronine residues-containing polypeptide fragments can be generated from Tg by proteases such as cathepsin D[24] or cathepsin B.[25] This enzymatic degradation, coupled with oxidant radicals-mediated cleavage, would lead to the facilitated release of thyroxine hormones.

It is possible to suppose that Cu^{2+}/ascorbic acid system can operate in the oxidative degradation of Tg *in vivo*. Concerning a potential source of copper ions *in vivo*, there are reports on the release of copper ions from proteins at sites of oxidative stress.[26,27] And the lowest concentration of ascorbic acid, in combination with 10 μM Cu^{2+}, required for the oxidative degradation of Tg during 2 h incubation is relatively low, being between 0.1 and 0.3 mM (data not shown). However, a recent report suggests that it is uncertain whether the pro-oxidant effect of ascorbic acid has a biological relevance.[28,29] The oxidative degradation could be more probable, if the concentration of Cu^{2+} increases via the alteration of physiological state or extraneous sources. However, this possibility needs further investigation, since there are antioxidant systems *in vivo*. Despite this, the Cu^{2+}-catalyzed oxidation of Tg might be one of the factors responsible for the degradation of Tg in thyroid tissue, where oxidant radicals are generated continuously.

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