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pH Profile of cytochrome *c*-catalyzed tyrosine nitration⋆

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In the present study, we investigated how cytochrome *c* catalyzed the nitration of tyrosine at various pHs. The cytochrome *c*-catalyzed nitration of tyrosine occurred in proportion to the concentration of hydrogen peroxide, nitrite or cytochrome *c*. The cytochrome *c*-catalyzed nitration of tyrosine was inhibited by catalase, sodium azide, cystein, and uric acid. These results show that the cytochrome *c*-catalyzed nitrotyrosine formation was due to peroxidase activity. The rate constant between cytochrome *c* and hydrogen peroxide within the pH range of 3–8 was the largest at pH 6 (37°C). The amount of nitrotyrosine formed was the greatest at pH 5. At pH 3, only cytochrome *c*-independent nitration of tyrosine occurred in the presence of nitrite. At this pH, the UV as well as visible spectrum of cytochrome *c* was changed by nitrite, even in the presence of hydrogen peroxide, probably *via* the formation of a heme iron–nitric oxide complex. Due to this change, the peroxidase activity of cytochrome *c* was lost.

Keywords: cytochrome *c*, nitrite, nitrotyrosine, pH, pseudo-peroxidase

INTRODUCTION

Nitrotyrosine has attracted much attention as a biomarker of oxidative (nitrative) stress in inflammatory, allergic, and other diseases (Greenacre & Ischiropoulos, 2001). Nitrotyrosine is formed *via* tyrosine nitration by peroxynitrite (Ischiropoulos *et al.*, 1992), which is generated by the fast reaction of superoxide anion and nitric oxide (Blough & Zafiriou, 1985). Peroxidases, such as myeloperoxidase (Sampson *et al.*, 1998), eosinophil peroxidases (Wu *et al.*, 1999), horseradish peroxidase, and lactoperoxidase (van der Vliet *et al.*, 1997), catalyze the nitration of tyrosine with hydrogen peroxide (H_2O_2) and nitrite. Moreover, nitrotyrosine is formed *via* the nitration of tyrosine by pseudo-peroxidase, such as hemoglobin (Grzelak *et al.*, 2001), myoglobin (Kilinc *et al.*, 2001), heme/iron (Bian *et al.*, 2003), and microperoxidase 8 (Ricoux *et al.*, 2001). Proteins that contain heme seem to have tyrosine nitration activity.

Cytochrome *c* is a hemeprotein and shows very low peroxidase activity (Radi *et al.*, 1991). Compound I-type intermediate, which is the active form of peroxidase, is produced by the reaction between cytochrome *c* and H_2O_2 (Barr *et al.*, 1996; Deterding *et al.*, 1998; Qian *et al.*, 2002; Lawrence *et al.*, 2003;

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Abbreviations: BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TBS-T, Tris/HCl buffer containing sodium chloride and Tween 20.

Chen *et al.*, 2004). The tyrosyl radical is formed by the cytochrome *c*-catalyzed oxidation of tyrosine (Barr *et al.*, 1996; Qian *et al.*, 2002; Chen *et al.*, 2004) as another peroxidase system (Totsune *et al.*, 1999). The peroxidase activity of cytochrome *c* is increased by its nitration, conformational change, and/or decomposition (Cassina *et al.*, 2000; Gębicka & Didik, 2003; 2005; Castro *et al.*, 2004; Jang & Han, 2006; Batthyány *et al.*, 2005). Recently, tyrosine nitration by cytochrome *c* was also reported (Chen *et al.*, 2004; Castro *et al.*, 2004). Moreover, cytochrome *c* and nitrated protein, which were detected histochemically, were positioned closely in neurons of the rat cerebral cortex after oxygen and glucose deprivation (Alonso *et al.*, 2002). Cytochrome *c*-catalyzed nitrotyrosine formation may involve some cellular events. To understand the ability of cytochrome *c* to act as a peroxidase, it is important to establish whether cytochrome *c* works as a peroxidase in biological systems. The kinetics and pH-dependency of cytochrome *c*-catalyzed tyrosine nitration reaction are useful indicators of such activity. They, however, have hardly been studied. Therefore, we investigated how cytochrome *c* catalyzed tyrosine nitration at various pH values.

MATERIALS AND METHODS

Materials. Cytochrome *c*, L-tyrosine, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 3-Nitro-L-tyrosine was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). H_2O_2, L-cysteine and sodium nitrite were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Peroxynitrite and diethylene triamine-N,N,N′,N′′,N′′-pentaacetic acid were obtained from Dojin (Kumamoto, Japan). Rabbit polyclonal anti-nitrotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY, USA). Solvents and other reagents were of the highest grade commercially available. Concentrations of cytochrome *c* and H_2O_2 were determined spectrophotometrically with their molar absorption coefficients at 410 nm and 240 nm, respectively (ε_{410} = 1.06 × 10^5 $M^{-1}cm^{-1}$ (Stellwagen, 1968), ε_{240} = 39.4 $M^{-1}cm^{-1}$ (Nelson & Kiesow, 1972)).

Effect of H_2O_2, nitrite, cytochrome *c*, or L-tyrosine on the cytochrome *c*-catalyzed nitrotyrosine formation. The standard reaction mixture (1.5 ml) contained 2.5 μM cytochrome *c*, 100 μM H_2O_2, 1 mM L-tyrosine, 1 mM nitrite, and 0.1 M potassium phosphate buffer, pH 7. To examine the effect of each factor on nitrotyrosine formation, various concentrations of H_2O_2, nitrite, L-tyrosine, or cytochrome *c* were used, as shown in Fig. 1. The reaction was started by the addition of cytochrome *c* at 37°C. After a 60 min incubation, 200 μl of reaction mixture was removed and 50 μl of 5 mM L-cysteine (final concentration: 1 mM) was added to stop the reaction. After filtration with Ultrafree®-MC (10,000 NMWL filter unit, MILLIPORE, Redford, MA, USA) at 2000 ***g*** for 3 h (4°C), 50 μl of filtrate was injected onto a reversed-phase HPLC equipped with a UV detector (type: L-7400, Hitachi, Tokyo, Japan) and autosampler (type: L-7200, Hitachi, Tokyo, Japan). Spherisorb® ODS2 (4.6 × 150 mm, 5 μm, Waters, Massachusetts, USA) and 50 mM potassium phosphate buffer, pH 3, containing 7% methanol were used as the column and mobile phase, respectively. The flow rate was 1.0 ml/min. Nitrotyrosine was monitored at 274 nm.

Effect of various inhibitors on cytochrome *c*-catalyzed nitrotyrosine formation. The control reaction mixture (250 μl) contained 2.5 μM cytochrome *c*, 100 μM H_2O_2, 1 mM L-tyrosine, 1 mM nitrite, and 0.1 M potassium phosphate buffer, pH 7. To examine the effect of various inhibitors on nitrotyrosine formation, 20 μg/ml catalase, 50 μg/ml superoxide dismutase (SOD), 5 mM sodium azide, 1 mM uric acid, 1 mM L-cysteine, 1 mM methionine, 50 mM dimethyl sulfoxide, 80 mM ethanol, or 50 mM mannitol was used. The reaction mixture was incubated at 37°C for 1 h. After the reaction was stopped and

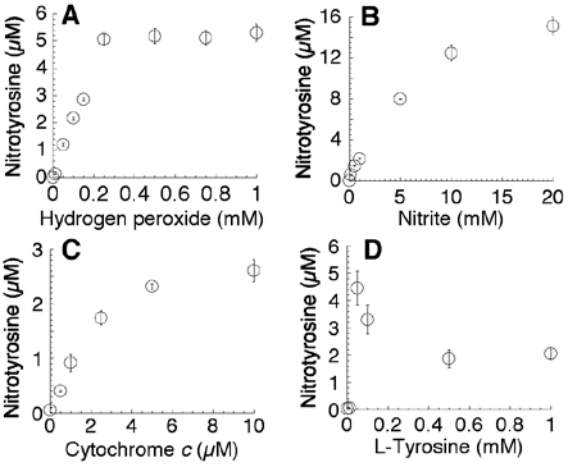

Figure 1. Effect of dose of hydrogen peroxide, nitrite, cytochrome *c*, or tyrosine on cytochrome *c*-catalyzed nitrotyrosine formation.
(A) Various concentrations of hydrogen peroxide were incubated with 2.5 μM cytochrome *c*, 1 mM L-tyrosine, and 1 mM nitrite in 0.1 M potassium phosphate buffer (pH 7) at 37°C for 1 h. (B) Various concentrations of nitrite were incubated with 2.5 μM cytochrome *c*, 100 μM hydrogen peroxide, and 1 mM L-tyrosine at 37°C for 1 h (pH 7). (C) Various concentrations of cytochrome *c* were incubated with 100 μM hydrogen peroxide, 1 mM L-tyrosine, and 1 mM nitrite at 37°C for 1 h (pH 7). (D) Various concentrations of L-tyrosine were incubated with 2.5 μM cytochrome *c*, 100 μM hydrogen peroxide and 1 mM nitrite at 37°C for 1 h (pH 7). Data are shown as the mean ± standard deviation of three separate experiments.

filtered as above, the filtrate (50 μl) was analyzed using a reversed-phase HPLC.

Kinetic study of the reaction between cytochrome *c* and H_2O_2 at various pHs. In this study, 0.1 M glycine/HCl buffer, pH 3, 0.1 M acetate buffer (pH 4 or 5), and 0.1 M potassium phosphate buffer (pH 6, 7, or 8) were used. Cytochrome *c* (2.5 μM) was incubated with or without 25–500 μM H_2O_2 at each pH (37°C; total volume: 1 ml). The absorbance at 408 nm was measured at 0, 10, 20, 30, and 60 min using a spectrophotometer (type: UV-1200, Shimadzu, Kyoto, Japan). Kinetic analysis as a pseudo-first order reaction was performed using the Kaleida Graph software (Synergy software, Reading, PA, USA).

Cytochrome *c*-catalyzed formation of nitrotyrosine at various pHs. Cytochrome *c* (2.5 μM) was incubated with 1 mM L-tyrosine, 1 mM nitrite, and 100 μM H_2O_2 at various pHs (37°C). An aliquot (200 μl) was removed from the reaction mixture at 0, 10, 20, 30, and 60 min, and the reaction was stopped, filtrated, and analyzed by HPLC as before. To examine the contribution of the chemical nitration reaction of tyrosine, 1 mM L-tyrosine was incubated with 1 mM nitrite and/or 100 μM H_2O_2 at various pHs (37°C).

Nitration of tyrosine residue in protein by cytochrome *c* at various pHs. Cytochrome *c* (1 mg/ml) was incubated with 1 mg/ml BSA in the presence of 1 mM nitrite, 100 μM H_2O_2, and 100 μM diethylene triamine-N,N,N′,N′′,N′′-pentaacetic acid at various pHs (37°C). Nitrated protein was detected by Western blot analysis with anti-nitrotyrosine antibody (Ogino *et al.*, 2001; 2002). After 60 min incubation, the reaction mixture was combined with an equal volume of non-reducing Laemmli buffer (125 mM Tris/HCl, pH 6.8, 5% SDS, 30% glycerol, and 0.01% bromophenol blue) and heated at 100°C for 5 min. Samples containing 2.5 μg protein were loaded onto 5–20% SDS/polyacrylamide gel (ATTO, Tokyo, Japan) and electrophoresis was performed. Proteins in gel were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) using a semi-dry method. The membrane was blocked with 5% nonfat dry milk in 20 mM Tris/HCl buffer, pH 7.7, containing 137 mM NaCl and 0.5% Tween 20 (TBS-T) for 1 h at room temperature, and then reacted overnight at 4°C with rabbit polyclonal anti-nitrotyrosine antibody (1:1000 dilution). After washing with TBS-T, the immunocomplexed membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (Dako, Carpinteria, CA, USA) (1:2000 dilution) for 1 h at room temperature. Immunoreactive proteins were visualized according to the enhanced chemiluminescence Western blot detection system (PerkinElmer, Boston, MA, USA).

Change in UV and visible spectrum of cytochrome *c* by nitrite at pH 3. The change in the UV and visible spectrum (300–500 nm and 450–650 nm) of cytochrome *c* (10 μM) in 0.1 M glycine/HCl buffer, pH 3, by nitrite and/or H_2O_2 at room temperature was monitored with a spectrophotometer. The following two conditions were examined.

1) The UV and visible spectrum of 10 μM cytochrome *c* was measured, then 1 mM nitrite (10 μl of 100 mM nitrite) was added to this solution and the UV and visible spectrum was measured. Finally, 100 μM H_2O_2 (10 μl of 10 mM H_2O_2) was added to the reaction mixture and the UV and visible spectrum was measured.

2) The UV and visible spectrum of 10 μM cytochrome *c* was measured, then 100 μM H_2O_2 was added to this solution and the UV and visible spectrum was measured. Finally, 1 mM nitrite was added to the reaction mixture and the UV and visible spectrum was measured.

The total volume of the reaction mixture was 1 ml.

Change in absorbance of cytochrome *c* by nitrite at 408 nm (pH 3). The change in the Soret band of cytochrome *c* (408 nm) by nitrite in 0.1 M glycine/HCl buffer, pH 3, at room temperature was followed photometrically. The following four conditions were examined.

1) Cytochrome *c* (10 μM) was incubated at room temperature.

2) Cytochrome *c* (10 μM) was incubated with 100 μM H_2O_2 at room temperature.

3) Cytochrome *c* (10 μM) was incubated with 100 μM H_2O_2 at room temperature for 10 min and then 1 mM nitrite was added to this solution.

4) Cytochrome *c* (10 μM) was mixed with 1 mM nitrite, then 100 μM H_2O_2 was added to this solution after measurement of the absorbance at 408 nm, and the reaction mixture was incubated at room temperature.

The total volume of the reaction mixture was 1 ml. The absorbance at 408 nm was measured every 10 min.

RESULTS

Cytochrome c-catalyzed nitration of L-tyrosine

Cytochrome *c* (2.5 μM) nitrated L-tyrosine in the presence of 100 μM H_2O_2 and 1 mM nitrite at pH 7 (37°C). The cytochrome *c*-catalyzed nitration of tyrosine occurred in proportion to the concentration of H_2O_2 (Fig. 1A), nitrite (Fig. 1B), and cytochrome *c* (Fig. 1C), but not that of L-tyrosine (Fig. 1D). As the L-tyrosine concentration was higher, more bityro-

sine would be formed, as in the case of myoglobin-catalyzed tyrosine nitration and oxidation (Herold, 2004).

Effect of various inhibitors on cytochrome *c*-catalyzed nitrotyrosine formation

To examine the reaction mechanism of tyrosine nitration by cytochrome *c*, various inhibitors were used (Fig. 2). Catalase (40 μg/ml), which reduces H_2O_2, thoroughly inhibited the cytochrome *c*-catalyzed tyrosine nitration reaction. SOD (50 μg/ml), which disproportionates the superoxide anion, showed a small increase in the nitrotyrosine formed. Sodium azide (5 mM), an inhibitor of hemeproteins, inhibited the reaction. Uric acid and cysteine, which react with compound I-type species, strongly suppressed the nitrotyrosine formation. However, methionine, dimethyl sulfoxide, ethanol, or mannitol did not affect this reaction. These results were comparable to those of the eosinophil-induced tyrosine nitration (our unpublished data) and were different from those of the peroxynitrite-induced nitration of tyrosine (Ogino *et al.*, 2001). Since iron shows peroxidase activity (Bian *et al.*, 2003), released and/or contaminated iron might nitrate tyrosine. EDTA, however, did not affect the cytochrome *c*-catalyzed tyrosine nitration (not shown).

pH profile of the rate constant between cytochrome *c* and H_2O_2, and that of free or protein-bound nitrotyrosine formation catalyzed by cytochrome *c*

The decrease in the absorbance at 408 nm (Soret band of cytochrome *c*) by H_2O_2 was followed at various pHs. Pseudo-first order rate constants (k_{obs}s) were calculated by fitting these data to the first order kinetics. Then, k_{obs} (s^{-1}) was plotted as a function of the concentration of H_2O_2 (M; 25-500 μM) to obtain the apparent second order rate constant (k_{app}) between cytochrome *c* and H_2O_2 as its slope. In the present study, k_{app} at pH 7 (25°C) was (0.42 ± 0.03) $M^{-1}s^{-1}$. This value was close to the reported value (0.24 $M^{-1}s^{-1}$) (Radi *et al.*, 1991). The k_{app}s between cytochrome *c* and H_2O_2 were (9.88 ± 2.55) $M^{-1}s^{-1}$, (5.28 ± 0.28) $M^{-1}s^{-1}$, (0.61 ± 0.14) $M^{-1}s^{-1}$, (1.10 ± 0.14) $M^{-1}s^{-1}$, (0.73 ± 0.18) $M^{-1}s^{-1}$, and (0.38 ± 0.03) $M^{-1}s^{-1}$ at pH 3, 4, 5, 6, 7, and 8, respectively (37°C; Fig. 3A). The k_{app} at pH 3 was the largest. Among pHs 5 to 8, the k_{app} at pH 6 was the greatest (Fig. 3B).

Under acidic conditions, chemical tyrosine nitration by nitrite occurs (Shigenaga *et al.*, 1997). In the present study, nitrite nitrated tyrosine (not shown), but more nitrotyrosine was formed in the presence of both nitrite and H_2O_2 at pH 3. Therefore, we investigated the contribution of chemical tyrosine nitration (nitration by nitrite and H_2O_2) to nitrotyrosine formation in the presence of cytochrome *c*. At pH 3, there was no difference in the amounts of nitrotyrosine formed between that in the presence and in the absence of cytochrome *c* (Fig. 4A). This means that the peroxidase-catalyzed nitration of tyrosine did not occur at pH 3. At pH 4, nitrotyrosine was also formed, even in the absence of cytochrome *c*,

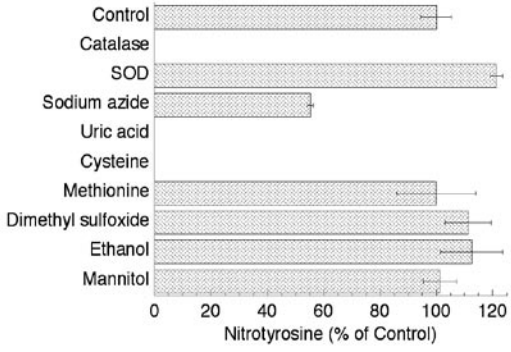

Figure 2. Effect of various inhibitors on the cytochrome *c*-catalyzed nitration of tyrosine in the presence of hydrogen peroxide and nitrite.
Various inhibitors were incubated with 2.5 μM cytochrome *c*, 100 μM hydrogen peroxide, 1 mM L-tyrosine, and 1 mM nitrite in 0.1 M potassium phosphate buffer, pH 7, at 37°C for 1 h. Catalase (20 μg/ml), SOD (50 μg/ml), sodium azide (5 mM), uric acid (1 mM), cysteine (1 mM), methionine (1 mM), dimethyl sulfoxide (50 mM), ethanol (80 mM), and mannitol (50 mM) were used as inhibitors. Data are shown as the mean ± standard deviation of three separate experiments.

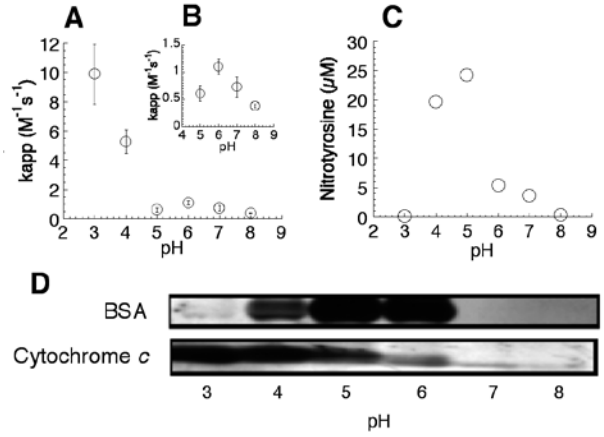

Figure 3. pH profile of apparent second order rate constant between cytochrome *c* and hydrogen peroxide, cytochrome *c*-catalyzed nitrotyrosine formation, and cytochrome *c*-catalyzed protein nitration.
(A, B) Cytochrome *c* (2.5 μM) was incubated with 100 μM hydrogen peroxide at various pHs (37°C). (C) Hydrogen peroxide (100 μM), L-tyrosine (1 mM), and nitrite (1 mM) were incubated with or without 2.5 μM cytochrome *c* at various pHs (37°C). The difference between the two conditions was plotted. (D) Cytochrome *c* (1 mg/ml) was incubated with 100 μM hydrogen peroxide, 1 mg/ml BSA, and 1 mM nitrite at various pHs (37°C). Data are shown as the mean ± standard deviation of three separate experiments (A, B, C). Representative results are shown of three separate experiments (D).

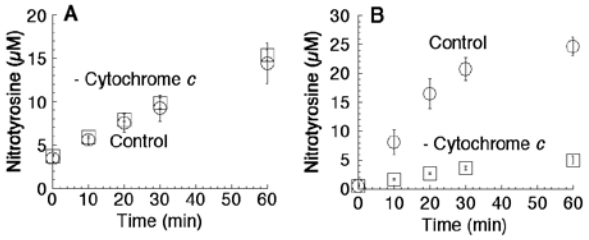

Figure 4. Tyrosine nitration *via* chemical reaction at acidic pH.
(A) Hydrogen peroxide (100 μM), L-tyrosine (1 mM), and nitrite (1 mM) were incubated with (open circle) or without (open square) 2.5 μM cytochrome *c* in 0.1 M glycine/HCl buffer, pH 3, at 37°C. (B) Hydrogen peroxide (100 μM), L-tyrosine (1 mM), and nitrite (1 mM) were incubated with (open circle) or without (open square) 2.5 μM cytochrome *c* in 0.1 M acetate buffer, pH 4, at 37°C. Data are shown as the mean ± standard de*via*tion of three separate experiments.

but its amount was extremely small compared with that in the presence of cytochrome *c* (Fig. 4B). At pH 5, small amounts of nitrotyrosine were formed under cytochrome *c*-free conditions (not shown). At pHs 6–8, the chemical nitration of tyrosine was not observed (not shown). Therefore, Fig. 3C expresses the difference between the amounts of nitrotyrosine formed in the presence and in the absence of cytochrome *c*. The amount of nitrotyrosine formed by the peroxidase activity of cytochrome *c* was the largest at pH 5. The nitration of BSA was the largest at pHs 5 and 6 (Fig. 3D). However, the nitration of cytochrome *c* was the greatest at pH 3 (Fig. 3D). At this pH, no cytochrome *c*-catalyzed nitration of free tyrosine was observed (Figs. 3C and 4A). Therefore, the cytochrome *c*-catalyzed protein nitration probably should not occur either. At pH 3, cytochrome *c* was nitrated *via* the chemical nitration reaction.

Inhibition of peroxidase activity of cytochrome *c* by nitrite at pH 3

The reason why the peroxidase activity of cytochrome *c* was not observed at pH 3 was examined. When nitrite was added to cytochrome *c*, the Soret band shifted to a higher wavelength (406 nm → 415 nm) and two novel peaks appeared (λ_{max}: 530 nm and 562 nm; Fig. 5A). When H_2O_2 was then added to this solution, the absorbance decreased, but no new peak appeared. In contrast, when H_2O_2 was first added to cytochrome *c*, the Soret band shifted to a slightly lower wavelength (Fig. 5B). When nitrite was then added to this reaction mixture, an absorption spectrum comparable to those in Figs. 5A 2 and 3 was observed. This spectrum was probably due to a heme–nitric oxide complex of cytochrome *c*, which had been reported previously (Orii & Shimada, 1978; Butt & Keilin, 1962). It is conceivable that heme-nitric oxide complex formation suppressed the peroxidase activity of cytochrome *c*. In a control system containing cytochrome *c* and H_2O_2, the absorbance at 408 nm decreased time-dependently (Fig. 6A). In the absence of H_2O_2, the absorbance at 408 nm did not decrease (Fig. 6A). Just after nitrite was added to cytochrome *c*, the absorbance decreased, owing to a change in the spectrum, as seen in Fig. 5A (Fig. 6B). In this case, the addition of H_2O_2 to the reaction mixture at 0 min did not change the absorbance at 408 nm except at the initial stage (Fig. 6B). Similarly, when nitrite was added to the solution after cytochrome *c* and H_2O_2 were incubated at room temperature for 10 min, no change in the absorbance was observed, except at the initial stage (Fig. 6C). These results show that the peroxidase activity of cytochrome *c* was suppressed by nitrite at pH 3, even with the pre-existence of H_2O_2.

DISCUSSION

The present study shows that cytochrome *c* catalyzes tyrosine nitration in the presence of H_2O_2 and nitrite at various pHs. The k_{app} between cytochrome *c* and H_2O_2 was the largest at pH 6 among pHs 5–8. On the other hand, the amount of nitrotyrosine formed by cytochrome *c* was the highest at pH 5. This difference would be due to the reaction rate of nitrotyrosine formation being determined by other reactions (e.g.: the reaction between compound I-like species and nitrite), but not by the reaction between cytochrome *c* and H_2O_2

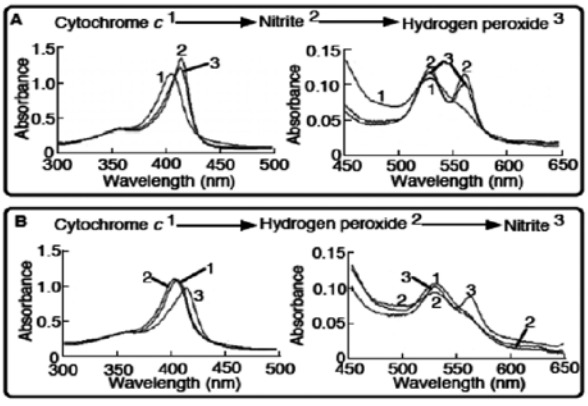

Figure 5. Change in cytochrome *c* spectrum by nitrite and/or hydrogen peroxide at pH 3.
(A) The spectrum (300–500 nm and 450–650 nm) of 10 μM cytochrome *c* in 0.1 M glycine/HCl buffer, pH 3, was recorded at room temperature (1). The spectrum was recorded after 1 mM nitrite was added (2), then 100 μM hydrogen peroxide was added, and the spectrum was recorded (3). (B) The spectrum (300–500 nm and 450–650 nm) of 10 μM cytochrome *c* in 0.1 M glycine/HCl buffer, pH 3, was recorded at room temperature (1). The spectrum was recorded after 100 μM hydrogen peroxide was added (2), then 1 mM nitrite was added and the spectrum was recorded (3). Representative results are shown of three separate experiments.

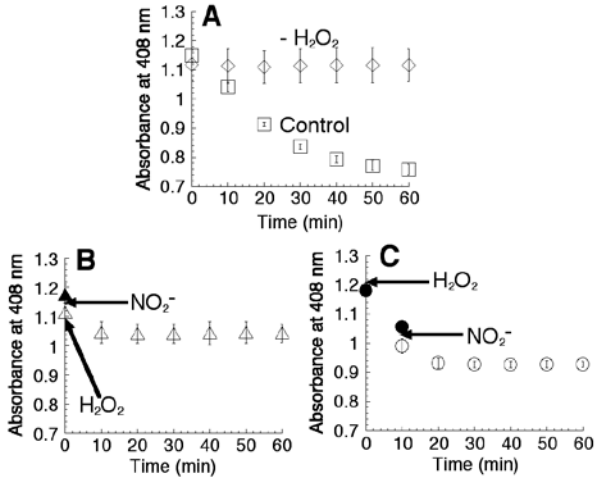

Figure 6. Inhibition of peroxidase activity of cytochrome *c* by nitrite at pH 3.
(A) Cytochrome *c* (10 μM) was incubated with or without 100 μM hydrogen peroxide in 0.1 M glycine/HCl buffer (pH 3) at room temperature (control, open square; H_2O_2, open diamond). (B) Cytochrome *c* (10 μM) was incubated with 100 μM hydrogen peroxide in 0.1 M glycine/HCl buffer, pH 3, for 10 min at room temperature (filled triangle) and then 1 mM nitrite was added (open triangle). (C) Nitrite (1 mM) was added to 10 μM cytochrome *c* in 0.1 M glycine/HCl buffer, pH 3, at room temperature (filled circle) and then 100 μM hydrogen peroxide was added (open circle). Arrows indicate the time point when chemical was added. Data are shown as the mean ± standard deviation of three separate experiments.

At pH 3, the k_{app} between cytochrome *c* and H_2O_2 was the largest. However, at this pH, cytochrome *c* did not show peroxidase activity in the presence of nitrite, and the cytchrome *c*–nitric oxide complex was observed spectrophotometrically. The formation of a myeloperoxidase–nitric oxide complex causes a loss of myeloperoxidase activity (Abu-Soud & Hazen, 2000). Therefore, it is conceivable that the peroxidase activity of cytochrome *c* was lost at pH 3 for the same reason. Another reason for the lack of the peroxidase activity of cytochrome *c* is not negligible. The reaction between nitrite (nitrous acid) and H_2O_2 takes place very rapidly under acidic condition (Anbar & Taube, 1954). Therefore, most of H_2O_2 should react with nitrous acid, but not with cytochrome *c*, and the active form of cytochrome *c* might hardly be formed at pH 3.

The nitration of cytochrome *c* occurred most easily at pH 3, although BSA was nitrated by cytochrome *c* most efficiently at pHs 5 and 6. One possibility was the dependency of pH-profile for the activity of peroxidases on the nature of the substrate. For example, the peroxidase activity of cytochrome *c* towards 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is the highest at pH 3.6–3.8 (Radi *et al.*, 1991). Another possibility was as follows. Only the chemical nitration reaction of free tyrosine occurred in the presence of nitrite at pH 3. Most of the tyrosine residues in cytochrome *c* might be located at locations where nitrite could easily access, probably due to the protein's conformational change of acidic pH. However, further study is needed to confirm the reason why cytochrome *c*, but not BSA, was nitrated at pH 3.

In the present study, the k_{app} between cytochrome *c* and H_2O_2 at pH 7 (25°C) was (0.42 ± 0.03) $M^{-1}s^{-1}$. This value was extremely low compared to that of myeloperoxidase (1.8×10^7 $M^{-1}s^{-1}$) (Marquez *et al.*, 1994) or eosinophil peroxidase (4.3×10^7 $M^{-1}s^{-1}$) (Furtmüller *et al.*, 2000). Thus, it appears difficult for cytochrome *c* to work as a peroxidase in biological systems judging only from its kinetics.

One might speculate on the role of nitrotyrosine formation by cytochrome *c* in biological systems. Cytochrome *c* is released from the mitochondrial membrane to the cytosol during apoptosis (Liu *et al.*, 1996; Kluck *et al.*, 1997). This cytochrome *c* release from electron transport induces more superoxide anion formation by electron leakage and H_2O_2 accumulation than in the normal state (Boveris & Cadenas, 1975; Forman & Kennedy, 1975; Jiang *et al.*, 2003). On the other hand, cytochrome *c* is bound to cardiolipin, which surrounds cytochrome *c* in mitochondria (Petrosillo *et al.*, 2003). When cardiolipin is oxidized during apoptosis, the affinity for cytochrome *c* is lost and cytochrome *c* is released into the cytosol (Nomura *et al.*, 2000; Petrosillo *et al.*, 2003). Phosphatidylserine is oxidized during Fas-mediated apoptosis (Kagan *et al.*, 2002). Thus, the apoptotic process involves oxidative stress. Cytochrome *c* oxidizes phosphatidylserine using H_2O_2 (Jiang *et al.*, 2003). A compound I-like species is formed more easily by interaction of cytochrome *c* and phosphatidylserine (Jiang *et al.*, 2003). This interaction detaches Met-80 form heme iron in cytochrome *c* (de Jongh *et al.*, 1995) and makes the peroxidase activity stronger. Moreover, nitrite can be formed in the vicinity of cytochrome *c* in mitochondria, since mitochondria contain nitric oxide synthase (Ghafourifar & Richter, 1997; Ghafourifar *et al.*, 1999; Ghafourifar & Cadenas, 2005; Giulivi *et al.*, 1998). Nitric oxide can induce apoptosis (Ghafourifar & Cadenas, 2005; Horteleno *et al.*, 2000). Therefore, tyrosine nitration by cytochrome *c* can occur during apoptosis. Nitration, conformational change, and/or the decomposition of cytochrome *c* cause on increase in the peroxidase activity of cytochrome *c* (Cassina *et al.*, 2000; Gębicka & Didik, 2003; 2005; Castro *et al.*, 2004; Batthyány *et al.*, 2005; Jang & Han, 2006). Cytochrome *c* may nitrate itself to increase the peroxidase activity in apoptosis, and then oxidize substances such as phosphatidylserine. Thus, the cytochrome *c*-catalyzed nitration of tyrosine may contribute to apoptosis.

In conclusion, cytochrome *c* catalyzes the nitration of free tyrosine and protein-bound tyrosine

by its peroxidase activity with H_2O_2 and nitrite at various pHs. It is thought that the cytochrome *c*-catalyzed tyrosine nitration is difficult to bring about in biological systems judging from the kinetics, but it may play a pivotal role in peculiar situations, such as apoptosis.

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