Appearance of Nitrite Reducing Activity of Cytochrome c upon Heat Denaturation

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The appearance of NO₂⁻ reducing activity of cytochrome c (Cyt c) upon heat denaturation was investigated with equine heart Cyt c. Denatured equine heart Cyt c (dCyt c), which was treated at 100°C for 30 min, had NO₂⁻ reducing activity in the presence of dithionite and methyleneviologen in an aqueous solution under anaerobic conditions. In contrast, hemoglobin and myoglobin had no such activity under the same conditions. Using spectroscopic methods, we found that the appearance of this activity in the Cyt c was due to the following intramolecular changes: unfolding of the peptide chain, exposure of the heme, dissociation of the sixth ligand methionine sulfur, and appearance of autoxidizability. The dCyt c catalyzed NO₂⁻ reduction to NH₄⁺ via ferrous-NO complexes and this reaction was a 6-electron and 8-proton reduction. Sepharose-immobilized dCyt c had activity similar strength to that in solution. The resin retained the activity after five uses and even after storage for 1 year. On the basis of these results, we concluded that Cyt c acquired a new catalytic activity upon heat treatment, unlike to other familiar biological molecules.

Key words: cytochrome c; heat denaturation; immobilized protein; nitrite reduction

During the natural nitrogen cycle, NO₂⁻ is reduced by nitrite reductase along two pathways. In dissimilatory reduction, also called denitrification, NO₂⁻ is used as a respiratory terminal substrate, which corresponds to oxygen in eukaryotes. There are two kinds of nitrite reductases, copper protein and cytochrome (Cyt) cd₁. The net reaction of these enzymes ends in the donation of one electron and two protons by the attached catalytic metal species. On the other hand, NAD(P)H-nitrite reductase and ferrodoxin-nitrite reductase, called assimilatory nitrite reductases, catalyze the reduction of NO₂⁻ to NH₄⁺, with the resultant NH₄⁺ being finally incorporated into amino acids. These enzymes have a siroheme and an Fe–S cluster as the prosthetic group. Ho et al. purified ferredoxin-nitrite reductase, the assimilatory nitrogen-cycle enzyme, from a red alga, *Porphyra yezoensis*. During the purification, they discovered that the crude extract after heat treatment reduced NO₂⁻, and found that the target molecule was denatured cytochrome (dCyt c)-553. Cyt c is a widely studied soluble hemoprotein the heme of which is coordinated with endogenous ligands, histidine and methionine. Although this protein was believed to function solely in electron transfer in the eukaryotic respiratory chain, it has been shown to be important in apoptosis and in diseases associated with oxidative stress. Oku et al. reported that equine heart Cyt c treated with heat or γ-rays reduces NO₂⁻ to NH₄⁺ via Cyt c-NO complexes. This unexpected phenomenon, in which a new catalytic activity appears after heat treatment, is opposite to the pattern of well-known biological molecules, which are generally inactivated by heat. The study that used dCyt c also reported that the molecule kept the color and remaining free NO₂⁻ unchanged in cured meat (ham, bacon, and sausage); the aim of the study had also been to avoid production of carcinogenic N-nitrosoamines that are produced by the reaction of NO₂⁻ with secondary amines from meat. However, it is not been known why the Cyt c was converted to a molecule that reduces NO₂⁻ on heat treatment. The preparation of dCyt c is easier than the synthesis of functionally corresponding nonorganic compounds, such as iron-substituted polyoxotungstate, an NADH analog, and 1,1’-dimethylferrocene, or the purification of nitrite reductases. The dCyt c was more stable than nitrite reductases (see below). For these reasons, the uses of dCyt c with NO₂⁻ reducing activity would be manifold. Therefore, we wanted to know more about dCyt c. Here, we report on the appearance of its NO₂⁻ reducing activity, suggest a pathway for
Materials and Methods

Preparation of hemoproteins. Equine heart Cyt c was purchased from Wako Pure Chemical Industries. Bovine blood hemoglobin and equine heart myoglobin were purchased from Sigma. To obtain the ferric forms, a portion of potassium ferricyanide was added to the protein solutions. After gel filtration on a column of Toyopearl HW-55F (2.5 × 80 cm; Tosoh), electrophoretically pure proteins were obtained.

Measurement of NO$_2^-$ reducing activity. A vial containing 0.4 ml of 100 mM phosphate buffer, pH 7.0, 0.4 ml of 10 mM sodium nitrite, 0.5 ml of 3 mM methyliodogen, and 0.4 ml of 15 μM (as heme) hemoprotein solution was sealed with a butyl rubber and an aluminum seal. The headspace of the vial was purged with argon gas. After incubation of the mixture at 37°C for 5 min, the reaction was started by the addition of 0.3 ml of 100 mM sodium dithionite dissolved in 50 mM sodium bicarbonate. The reaction was done at 37°C for several minutes. Part of the reaction mixture was put in a test tube at intervals of several minutes, and the tube was vigorously shaken until the color of reaction mixture was completely changed from blue to colorless. For the detection of NO$_2^-$, to 50 μl of the reaction mixture, 1,950 μl of water, 1 ml of 1% sulfanilamide, 1 ml of 0.02% N-1-naphthylethylenediamine, and 1 ml of water were added, in that order. After incubation of the mixture at room temperature for 20 min, the optical density at 540 nm was measured. One unit of activity was defined as a reduction of 1 μmol of NO$_2^-$ per minute. The $k_{cat}$ was calculated from Lineweaver–Burk plots. Other slight modifications of the methods are mentioned in the figure legends.

For detection of released NH$_4^+$, to 100 μl of the reaction mixture, 1,900 μl of water, 1 ml of 50 mg% sodium nitroprusside in 10% phenol, 1 ml of 0.06% sodium hypochlorite solution dissolved in 0.1 M sodium hydrogen phosphate-0.25 M sodium hydroxide, and 1 ml of water were added. After incubation of the mixture at room temperature for 60 min, the optical density at 630 nm was measured. Nitrous oxide was detected by gas chromatography on a Shimadzu GC-14B apparatus equipped with a Porapak Q column.

Redox titration studies. Redox titration studies were done under anaerobic conditions, under a continuous stream of argon, in sodium phosphate buffer, pH 7.0, at 25°C. Duroquinone, 1,4-naphthoquinone, phenazine ethosulfate, naphthoquinone-4-sulfonate, 2,3,5,6-tetramethyl-p-phenylenediamine, and methylene blue were used as reagents mediators. By injection of small volumes of dithionite and ferricyanide solutions, reduction and oxidation of the proteins, respectively, were brought about. Potentials were measured with a Beckman Φ310 pH meter equipped with a Microelectrodes MI-800-410 ORP electrode. Optical spectra were recorded throughout the titration on a Hitachi U-3000 spectrophotometer. These results were analyzed with a theoretical curve based on the Nernst equation ($n=1$): $E=E^0+(RT/nF) \ln ([\text{ferric}]/[\text{ferrous}])$, where $E$ is redox potential, $E^0$ is standard redox potential, $R$ is gas constant, $T$ is Kelvin temperature scale, $n$ is the number of electrons transferred per mole of reactants, $F$ is Faraday’s constant, and [ferric] and [ferrous] are the molar concentration of ferric and ferrous Cyt c, respectively.

Preparation of Sepharose-immobilized dCyt c. Sepharose-immobilized dCyt c was prepared by the method of Shin and Oshino. CNBr-Sepharose 4B was purchased from Pharmacia. One milligram of CNBr-Sepharose 4B was mixed with 10 ml of 1 mM HCl, and washed several times with the same solution. The resin was suspended in 5 ml of reaction buffer (0.1 M sodium bicarbonate-0.5 M sodium chloride) and treated with 5 ml of 1 mg/ml dCyt c, previously dialyzed with reaction buffer, at room temperature for 2 h. The resin bound dCyt c was then treated with 10 ml of blocking buffer (0.5 M ethanolamine-0.05 M sodium bicarbonate-0.25 M sodium chloride) at room temperature for 1 h. Finally, the immobilized dCyt c was washed with 0.1 M Tris-chloride, pH 7.5-0.5 M sodium chloride. The binding rate of the dCyt c to CNBr-Sepharose 4B was 98.98%.

Preparation of acrylamide-immobilized dCyt c. The dCyt c was mixed into 16.5% acrylamide gel, with the final concentration of the heme being 0.67 mg/ml. After addition of 100 mM sodium phosphate, pH 7.0, to obtain a final volume of 8 ml, the gel was disrupted with a spatula. The rate of inclusion of the dCyt c in acrylamide was 99.61%.

Spectroscopes. Visible spectra were monitored with a Milton Roy Spectronic 3000 Array. Circular dichroism (CD) spectra were measured at 25°C with a JASCO J-700 spectropolarimeter in a 2-mm light pass quartz cuvette. An electron spin resonance (ESR) spectrum was measured at cryogenic temperatures with a JEOL ESR spectrometer, JES-FA200.

Results and Discussion

Physicochemical properties of dCyt c

The activity of the native proteins, Cyt c, hemoglobin, and myoglobin, was nearly the same,
but when denatured, only Cyt c had a higher activity (5.4-fold that of the native form) (Fig. 1). The heme of Cyt c formed a His–Fe–Met octahedral coordination and a thioether bond with two cysteine residues of the peptide chain, and that of hemoglobin or myoglobin bound only the histidine residue as a fifth ligand. Hemin, hematin, and their imidazole complexes did not have such activity (Yamada, et al., unpublished results). The typical sequence motif –CXXCH– may be necessary for Cyt c to have the NO\textsubscript{2} reducing activity after denaturation.

The $k_{\text{cat}}$ values of native and denatured Cyt c were $13.3 \times 10^{-3}$ and $71.3 \times 10^{-3}$ sec\textsuperscript{-1}, respectively. The $k_{\text{cat}}$ of dCyt c was 1/200 that for ferredoxin-nitrite reductases.\textsuperscript{18} Ferredoxin-nitrite reductases have siroheme as the reaction center, the proximal ligand of which is a cysteine-sulfur shared with the 4Fe–4S cluster; the prosthetic groups are too electron-rich and push the electrons more strongly than histidine-coordinated heme enzymes.\textsuperscript{19} On the basis of the structure of sulfite reductase, which has a primary sequence, prosthetic group, and catalytic properties similar to ferredoxin-nitrite reductases, the active site is surrounded with positively charged residues that contribute to proton delivery to the substrate.\textsuperscript{20} The $k_{\text{cat}}$ of dCyt c being than that of ferredoxin-nitrite reductases probably arises from the differences in the proximal ligand and the environment of the heme.

This activity of Cyt c gradually increased with temperature and was maximum at 100°C (Fig. 2(A)). The curve in the figure, relating the temperature of treatment to the appearance of the activity, resembles the heat denaturation curve obtained by CD spectra for equine heart Cyt c.\textsuperscript{21} The ellipticity at 222 nm begins to disappear at 80°C, and the resultant denaturation curve gives a midpoint of melting temperature $T_{1/2}$ of 85.5°C. Perhaps the appearance of the activity in Cyt c is parallel to the unfolding of the peptide chain (see next section).

The optimum temperature for the activity of the dCyt c was 100°C (Fig. 2(B)). Nitrite reductases have

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* In the reference, the results for plotting of a heat denaturation obtained by CD spectra for equine heart Cyt c is given for comparison with that of Hydrogenobacter Cyt c-552.

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**Fig. 1.** NO\textsubscript{2} Reducing Activity of Native and Denatured Hemoproteins.

To prepare the denatured hemoproteins, purified hemoprotein solutions were treated at 100°C for 30 min and transferred immediately onto ice. After centrifugation, the supernatants were collected and concentrated to 15 μM heme. Cyt c, ◦; hemoglobin, ▽; myoglobin, □. Closed symbols indicate the corresponding denatured proteins. Results when the NO\textsubscript{2} concentration was 2 mM were taken to be 100%.

**Fig. 2.** Effects of Temperature of Treatment (A) and Reaction Temperature (B) of dCyt c, with Arrhenius Plot (C).

(A) A Cyt c solution (15 μM) was treated at different temperatures for 30 min and transferred immediately onto ice. After monitoring of the course of NO\textsubscript{2} reduction at 37°C for 25 min, the initial velocities for each sample were calculated and plotted. (B) dCyt c was prepared by treatment at 100°C for 30 min. The changes at each temperature were measured, and the initial velocities were plotted. (C) Arrhenius plot of (B).
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an optimum temperature at 50–60°C, above which they undergo denaturation. For example, NAD(P)H-nitrite reductase from *Azotobacter agile* and other ferredoxin-nitrite reductases lose activity after heat treatment at 50 and 60°C, respectively.18,22) The optimum temperature of dCyt c matches this because the sample was already treated at 100°C. In this study, the reaction mixture was in aqueous solution, and measurement could not be done at temperatures higher than 100°C. However, dCyt c may act at higher temperatures when in a different solvent. The activation energy of the dCyt c calculated from an Arrhenius plot was 51.5 kJ/mol (Fig. 2(C)), nearly the same as the activation energy (50 kJ/mol) of nitrite reductase from *Phormidium laminosum*;23) in other words, the thermodynamic catalytic ability of dCyt c was equal to that for nitrite reductases. dCyt c had an optimum pH of 7 for NO₂⁻ reducing activity of 7 (Fig. 3), and the range of active pH resembled that of nitrite reductases.18)

Appearance of NO₂⁻ reducing activity of Cyt c by heat denaturation

CD spectra of the dCyt c showed a lower mean residue ellipticity at 222 nm than that of the native protein (Fig. 4). This difference indicates that the helices of dCyt c were unfolded. The redox potentials of the native and dCyt c were 260 and 0 mV, respectively (Fig. 5). The heme is surrounded by hydrophobic amino acids, which create a hydrophobic patch in Cyt c, and its redox potential is high in biological redox components.24,25) A study of the redox potential and hydrophobicity of Cyt c concluded that water molecules stabilize the ferric heme, that is, that the redox potential of the heme in hydrophilic conditions was low.26) From our results and this information, we conclude that the heme in the dCyt c probably is exposed when the solvent is water.

In the visible spectra, a peak at 695 nm from the ferric form, which indicates axial coordination between the heme-iron and methionine-sulfur,27) disappeared in dCyt c, so that the heme probably was in a 5-coordinated form (Fig. 6(A)). The crystal structure
of Cyt c2-NH4+ complex showed that the detachment of the methionine axial ligand was a basic step in folding/unfolding process.28) The results suggest we can assume that the methionine ligand was dissociated from the heme of Cyt c by heat denaturation. If so, NO2– should bind easily to the sixth position of the heme. This result agrees with that of a chemical modification study that found that the association rate of NO as an exogenous ligand to Met 80-carboxymethylated Cyt c is greater than that in the native form.29)

After the addition of dithionite to dCyt c, the spectra showed a change from the ferrous form to the ferric form (Fig. 6(B)). This change suggests that dCyt c was autooxidizable and did not bind with oxygen different from hemoglobin and myoglobin. Perhaps dCyt c quickly donates an electron to the substrate, NO2–, so that the ferrous heme releases an electron, thus changing to the ferric as the resting form. Wallace and Clark-Lewis characterized various Cyts c in which the sixth ligand was changed to various amino acids in a semi-synthetic way.30) The M80A Cyt c mutant (Met 80 was replaced by alanine) did not have 6-coordination, and was autooxidizable. Our observation agrees well with theirs.

In summary, the following intramolecular changes occur in Cyt c denatured by heat: (1) unfolding of the peptide chain, (2) dissociation of the sixth ligand methionine from heme-iron, (3) exposure of the heme to the solvent, water, (4) acquired autoxidizability of the heme, with the concomitant appearance of NO2– reducing activity.

Reaction pathway for NO2– reduction in dCyt c

DcCyt c reduced NO2– to NH4+ under anaerobic conditions, such as in nitrogen or argon, and the rate of conversion was 100% (Fig. 7). In the presence of air or oxygen, the rates were 80% and 60%, respectively, with the remaining NO2– being converted to N2O (data not shown). This mechanism producing N2O under aerobic conditions is not understood in detail. Reduction of NO2– directly to NH4+ in ferredoxin-nitrite reductase is through three rounds of 2-electrons donations.31) The 4Fe–4S cluster in nitrite reductase can mediate the electron delivery between ferredoxin and siroheme. However, this is not applicable to dCyt c, which delivers only one electron. In the reaction mixture used in our study, the 6-electron reduction would be due to higher accessibility of the exposed heme of dCyt c to methylviologen, an electron donor.

Native Cyt c did not react with NO2– at pH 7 in either ferric or ferrous form, as discussed in previous reports.32,33) The addition of NO2– at pH 7, however, changed the visible spectrum of ferrous dCyt c (Fig. 8). The Soret peak shifted to 415 nm from 412 nm, and intensities of the α-peak (550 nm) and β-peak (520 nm) increased. These increases were probably due to the formation of NO2––Fe–His coordina-
Electron Spin Resonance Spectrum of dCyt c during the NO₂ "Reducing Reaction."

The reaction mixture was prepared as stated in Materials and Methods except that the concentration of dCyt c was 1 mM. The spectrum was monitored under liquid nitrogen. The resultant hyperfine structure near and at \( g = 2 \) corresponded to ferrous-NO complex.

From these results, we conclude that ferrous-NO complexes were an important intermediate in the NO₂ reducing reaction of dCyt c.

In conclusion, dCyt c in ferrous form reduces NO₂ completely to NH₄⁺ under anaerobic conditions via ferrous-NO complexes, intermediate in the reaction. This NO₂ reducing reaction is a 6-electron and 8-proton reduction.

**Immobilized dCyt c, its NO₂ reducing activity, stability, and reuse**

We mentioned above that dCyt c had NO₂ reducing activity, but dCyt c included in an aqueous reaction mixture for the detoxification of NO₂ is difficult to recycle. With this in mind, we tried to improve the reusability and stability of the dCyt c, and prepared dCyt c immobilized with CNBr-Sepharose or acrylamide. Sepharose-immobilized dCyt c had nearly the same activity as the denatured soluble protein (Fig. 10). In these circumstances, the peptide chain and the heme of dCyt c are exposed to the solvent in the same way as for a soluble protein. With inclusion in acrylamide, the activity was lower than that with Sepharose immobilization because dCyt c is surrounded by a matrix, and the matrix prevents its contact with the substrate or electron donor.

Both of the immobilized proteins retained activity even after storage at 4°C for 1 year (Table 1). With acrylamide inclusion, the activity remaining after 5 uses was 58.7%. The loss of activity could be due to the leakage of dCyt c from the acrylamide matrix. The Sepharose-immobilized dCyt c, which was washed with 100 mM phosphate-Na buffer, pH 7.0, after every use, had the same level of activity as that of a freshly prepared batch (Table 2). The resin did not lose activity even after repeated use. The covalent bond between dCyt c and the Sepharose resin was stable.
In this study, we offer a novel interpretation of the new catalytic property acquired by dCyt c. dCyt c has NO\textsubscript{2} reducing activity, which was thermodynamically equal to that of nitrite reductases, and had improved stability for high reaction temperatures and long-term storage. These properties arose from the molecule being denatured.

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