Folding Character of Cytochrome *c* Studied by *o*-Nitrobenzyl Modification of Methionine 65 and Subsequent Ultraviolet Light Irradiation†

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ABSTRACT: The protein folding character of cyt *c* was studied with the use of a photocleavable *o*-nitrobenzyl derivative of Met65 (NBz-Met65). For the NBz-Met65 cyt *c*, the Soret absorption band slightly blue shifted compared with the unlabeled cyt *c*, the 695 nm absorption band related to the Met80 sulfur ligation to the heme iron disappeared, and its resonance Raman spectrum was characteristic of a six-coordinate low-spin species, all characters demonstrating coordination of a non-native ligand, probably a histidine, instead of Met80 to the heme iron. The far-UV circular dichroism (CD) spectrum of cyt *c* was altered, and the transition midpoint concentration value of guanidine hydrochloride (GdnHCl) for unfolding the protein decreased by 0.9 M by the modification, which showed perturbation of the structure and decrease in protein stability, respectively. With irradiation of 308 nm laser pulses on the NBz-Met65 cyt *c*, the Soret absorption band slightly red shifted, the 695 nm absorption band appeared, and the CD spectrum shifted toward that of the native protein, which demonstrated recovery of the methionine heme coordination and the native protein structure, due to reconversion of NBz-Met65 to unlabeled methionine. A fast phase was detected as a change in Soret absorbance with a rate constant of 21 000 ± 4000 s^{-1} during refolding of cyt *c* initiated by irradiation of a 308 nm pulse on the NBz-Met65 cyt *c* in the presence of 2 M GdnHCl. The observed rate constant corresponded well with that reported by the tryptophan fluorescence study [Shastry, M. C. R. S., and Roder, H. (1998) *Nat. Struct. Biol. 5*, 385−392]. The intermediate decayed with a rate constant of 90 ± 15, followed by another phase with a rate constant of 13 ± 3 s^{-1}, and was not seen in the absence of GdnHCl.

Cytochrome *c* (cyt *c*)[1] is frequently used as a model for protein folding studies, due to its relatively small size and covalently bound heme, whose properties are sensitive to its environment and spin state. The axial ligands of the heme iron for native cyt *c* are Met80 and His18 (*1*, *2*), whereas a non-native histidine instead of Met80 coordinates to the iron when the protein is unfolded with a high denaturant concentration at neutral pH (*3*, *4*). Both His26 and His33 have been implicated as the non-native histidine (*4*, *5*), but it has been suggested recently by cyt *c* mutant studies that His33 is the dominant sixth heme ligand in the unfolded state (*3*). At low pH, one or both of the axial ligands are replaced by water molecules (*6*, *7*).

The heme and its axial ligands of cyt *c*, which are essential for structural stabilization (*8*) and function (*9*) of the protein, affect its folding dynamics (*3*, *10*−*16*). Among the three kinetic phases observed as changes in Soret absorbance and tryptophan fluorescence during folding of cyt *c*, the intermediate phase with a time constant of about 240 ms disappeared at low pH, whereas the intermediate and slow phases both disappeared by addition of an excess of an exogenous imidazole, indicating that the bis-histidine-coordinated form of the unfolded protein creates a kinetic barrier during folding of the protein (*10*). Changes in the heme axial ligands during folding of cyt *c* have been studied in detail for the time scale from 100 μs to 40 ms by resonance Raman (RR) spectroscopy combined with a rapid mixer (*13*−*15*). These results indicated that replacement of the axial heme ligand of cyt *c* would occur even after a few milliseconds during its folding process. Although the stopped-flow method combined with various types of spectroscopies (*11*, *17*−*20*) and the hydrogen exchange labeling method (*21*) are useful techniques and widely used for studying protein folding characters, the early event of protein folding is difficult to follow by these methods, where the time resolution is limited by the mixing dead time of a few milliseconds. For example, about 44% of the α-helical signal of native ferric cyt *c* occurred within the dead time of the stopped-flow circular dichroism (CD) measurement, indicating that a significant amount of the secondary structure was

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[1] Abbreviations: cyt *c*, cytochrome *c*; NBz-Met65, *o*-nitrobenzyl derivative of Met65; GdnHCl, guanidine hydrochloride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism; RR, resonance Raman.

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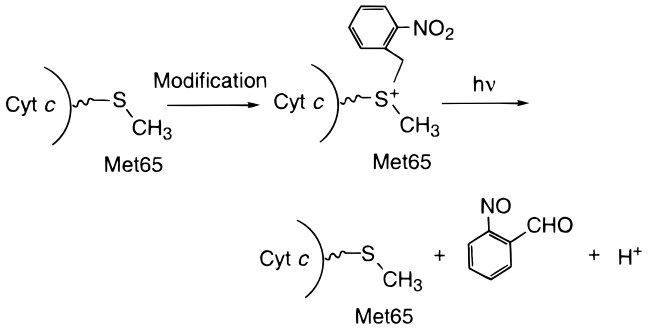
formed within 4 ms (*19*). Recently, attempts have been made to develop new methods, such as photoinduced heme reduction (*22*−*24*), heme−CO ligand photolysis (*25*), and rapid mixing (*13*−*16*, *26*, *27*) methods, which would elucidate the fast refolding character of cyt *c*, although these methods were limited to heme-containing proteins or reactions which occur longer than 45 μs.

Chemical modification has been used to study the molecular recognition and structural properties of proteins including cyt *c* (*28*−*30*). Small structural perturbation of cyt *c* has been reported for carboxymethylation of Met65 (*31*), whereas chemical modification of Met80 with iodoacetic acid has been shown to affect the protein stability (*32*). It has been shown that creatine kinase with its thiol groups modified by 5,5′-dithiobis(2-nitrobenzoic acid) had been partially unfolded and could be refolded and reactivated in the presence of dithiothreitol (*33*, *34*). A photocleavable disulfide bond was introduced into a model peptide, and re-formation of its secondary structure was observed by irradiation of a UV light (*35*). *o*-Nitrobenzyl derivatives are also photoreactive (*36*) and have been utilized to study the kinetics of neurotransmitter receptors (*37*, *38*). Recently, the *o*-nitrobenzyl group was introduced into a Ras protein to study its molecular recognition character (*39*).

To investigate the folding character of cyt *c* and to develop a general method for studying protein folding characters, we introduced a photocleavable *o*-nitrobenzyl group into cyt *c* by chemical modification. Alkylation of the methionine residue was performed, since cyt *c* has only two methionine residues and the thioether group of methionine can be selectively alkylated at low pH. Cyt *c* with an *o*-nitrobenzyl derivative of Met65 (NBz-Met65) was partially unfolded and started to refold by reconversion of NBz-Met65 to unlabeled Met65 by irradiation of a UV light according to the reaction:



The time-resolved absorption change with irradiation of a UV light on the NBz-Met65 cyt *c* is also studied with the use of the laser photolysis reaction.

EXPERIMENTAL PROCEDURES

Synthesis and Purification of Cyt c with o-Nitrobenzyl-Modified Methionine. Horse heart cyt *c* was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chemical modification of cyt *c* methionines to their *o*-nitrobenzyl derivatives was performed by mixing cyt *c* (90 mg) in 50 mM glycine hydrochloride buffer (6 mL), pH 1.5, with *o*-nitrobenzyl bromide (10 mg) in dimethylformamide (0.6 mL). The mixed solution was stirred for 15 min at 50 °C under a nitrogen atmosphere and in the dark to avoid sulfoxide formation and photocleavage, respectively. Urea and $(NH_4)_2SO_4$ were added to the *o*-nitrobenzyl-modified cyt *c* solution to final concentrations of 3 and 2 M, respectively. The modified cyt *c*'s were separated using a Phenyl-Toyopearl column (15 × 2.5 cm, volume 23 mL) in the dark at 4 °C with a flow rate of 1 mL/min. Unlabeled cyt *c* eluted from the column with 10 mM Tris-HCl buffer, pH 7.4, containing 2 M $(NH_4)_2SO_4$ and 3 M urea, while modified cyt *c*'s attached to it. For purification of modified cyt *c*'s, a gradient of the $(NH_4)_2SO_4$ concentration from 2 to 1 M (300 mL of 2 M + 200 mL of 0 M) with 10 mM Tris-HCl buffer, pH 7.4, containing 3 M urea was performed. At the final stage, 10 mM Tris-HCl buffer, pH 7.4, containing 3 M urea without $(NH_4)_2SO_4$ was used. Potassium ferricyanide was added to each modified cyt *c* fraction in 50 mM Tris-HCl buffer, pH 7.4, and the oxidant was subsequently removed using a DEAE-Sephacel column. To confirm that the modified protein was monomeric, gel filtration of unlabeled and modified cyt *c*'s was performed in the presence and absence of 2 M GdnHCl on a Sephadex G-100 column (25 × 1 cm, volume 20 mL) with 10 mM Tris-HCl buffer, pH 7.4, in the dark at 4 °C with a flow rate of 0.5 mL/min.

Cleavage of Cyt c at Met65 with CNBr. Cyt *c* is preferentially cleaved at Met65 by the reaction with CNBr under the reported condition (*40*). CNBr (50 equiv to cyt *c*) in 98% formic acid (0.7 mL) was added to unlabeled or modified cyt *c* in 50 mM Tris-HCl buffer, pH 7.4. The solutions were stirred for 24 h at room temperature under a nitrogen atmosphere in the dark. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis was performed for the solutions before and after the reaction with CNBr which were dialyzed with 50 mM Tris-HCl buffer, pH 7.4. The analysis was performed using a 12% separation gel with a Tris−glycine buffer. The obtained gel was stained with CBB R-250.

Absorption and CD Spectral Measurements. Ferric unlabeled and NBz-Met65 cyt *c* were dissolved in 50 mM Tris-HCl buffer, pH 7.4, or 20 mM sodium phosphate buffer, pH 7.2. Photolysis of NBz-Met65 was performed by irradiation of 100 shots of laser pulses at 308 nm (3 μs, 1 Hz, about 8 mJ per pulse per cm^2) obtained with a XeCl excimer laser (Lambda Physik, LPX120). After UV irradiation, the small amount of photoreduced cyt *c* was oxidized by adding potassium ferricyanide in 50 mM Tris-HCl buffer, pH 7.4, and subsequently removing the oxidant with a DEAE-Sephacel column for absorption measurements at 250−600 nm. To obtain the proportion of recovery of the unlabeled protein by irradiation of UV pulses on the NBz-Met65 cyt *c* in the presence of 2 M guanidine hydrochloride (GdnHCl), the intensities of the 695 nm absorption band and the CD spectra of the unlabeled protein and those before and after UV irradiation on the NBz-Met65 cyt *c* were compared in the presence of 2 M GdnHCl, whereas the absorption spectra in the Soret region were compared after removing GdnHCl and reoxidizing the small amount of photoreduced protein. Absorption and CD spectra were recorded with a Shimadzu UV-3101PC spectrometer and a Jasco J-720 spectropolarimeter, respectively, in quartz cells with path lengths of 10 and 0.5 mm, respectively. All measurements were carried out at 10 °C.

RR Measurements. Ferric unlabeled and NBz-Met65 cyt *c*'s (20 μM) were dissolved in 50 mM Tris-HCl buffer, pH 7.4. Raman scattering was excited at 406.7 nm with a Kr^+

laser (Spectra Physics, 2060) and detected with a CCD detector (Princeton Instruments, PI-CCD) attached to a single polychromator (Ritsu Oyo Kogaku, DG-1000). The laser power was adjusted to 10 mW, and the diameter of the laser beam was focused to 50 μM at the sample point. Raman shifts were calibrated with acetone, and the accuracy of the peak positions of the Raman bands was ± 1 cm^{-1}. Measurements were carried out at room temperature with a flow cell (600 μm $\times$ 600 μm).

Time-Resolved Absorption Measurements. Ferric NBz-Met65 cyt *c* (8 μM) was dissolved in 50 mM Tris-HCl buffer, pH 7.4, in the presence of 2 M GdnHCl or in the absence of it and placed in a quartz cuvette (path length, 10 mm). Photolysis of NBz-Met65 cyt *c* was initiated by the XeCl excimer laser pulse at 308 nm (3 μs, about 12 mJ per pulse per cm^2). Time-resolved absorption changes were measured at 10 °C with a light obtained from a halogen lamp which was introduced into the cuvette orthogonal to the laser pulse. The absorption changes were recorded on a digital oscilloscope (Lecroy, LC574A), which received voltage signals from the photomultiplier attached to a monochromator (Unisoku, RSP-601-03). The sample solution in the cuvette was renewed after each pulse. Least-squares exponential fits were performed for the time-resolved absorption data using Igor Pro version 3.13 (WaveMetrics).

RESULTS AND DISCUSSION

Purification of NBz-Met65 Cyt c and Identification of Met65 Modification. Specific chemical modification of a methionine thioether group has been reported for modification of cyt *c* with iodoacetic acid at pH 1.5, due to protonation of the reactive side chains of other residues, such as lysine and histidine, at low pH (*32*). Likewise, *o*-nitrobenzyl bromide would specifically react with the thioether group of methionine at pH 1.5. The methionine-modified cyt *c*'s which absorbed on the hydrophobic column were separated into three well-resolved fractions by performing a gradient of the $(NH_4)_2SO_4$ concentration from 2 to 0 M with 10 mM Tris-HCl buffer, pH 7.4, containing 3 M urea (*41*). The two fractions, which eluted from the column on decreasing the $(NH_4)_2SO_4$ concentration, both contained one *o*-nitrobenzyl methionine derivative per molecule, as estimated by comparison of the absorbance increase around 270 nm by the modification with the intensity of the 270 nm absorption band of the *N*-acetylmethionine *o*-nitrobenzylsulfonium chloride in the same buffer. The most hydrophobic modified cyt *c* fraction which started to elute with the buffer without $(NH_4)_2SO_4$ should have both methionines modified. Actually, the intensity increase of the 270 nm absorption band of this fraction indicated that the protein contained two *o*-nitrobenzyl-modified methionines per molecule.

CNBr is well-known to preferentially react with the side chain of Met65 and cleave the protein for unlabeled cyt *c* (*40*), while it would not react with the protein when the side chain of Met65 is derivatized. Unlabeled cyt *c* and the two single-methionine-modified cyt *c* solutions were subjected to the CNBr reaction and subsequent SDS−PAGE analysis. The results of the SDS−PAGE analyses for the unlabeled and two single-methionine-modified cyt *c*'s are shown in Figure 1. CNBr-treated unlabeled cyt *c* exhibited a smaller molecular weight than unlabeled cyt *c* due to cleavage at Met65 by the CNBr reagent (Figure 1, lane b), while the amount of the cleaved protein decreased significantly for the *o*-nitrobenzyl-modified cyt *c* of the latter-eluted fraction (Figure 1, lane f). These results indicated that the *o*-nitrobenzyl-modified methionine for the protein of the latter-eluted fraction was Met65. However, the protein of the former-eluted fraction of the two single-methionine-modified cyt *c*'s reacted with CNBr (Figure 1, lane d). Since *o*-nitrobenzyl-modified cyt *c* of the former-eluted fraction contained one *o*-nitrobenzyl methionine other than Met65, the protein should be modified at the position of another methionine, Met80. Among the three modified cyt *c*'s, the NBz-Met65 cyt *c* was used in this study. However, the NBz-Met65 cyt *c* was estimated to have a molecular weight similar to that of the unlabeled protein as judged by gel filtration in the presence and absence of 2 M GdnHCl, which suggested that the NBz-Met65 cyt *c* was monomeric under both conditions.

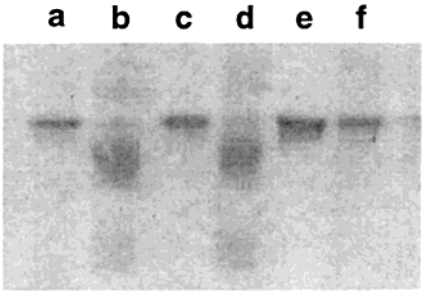


FIGURE 1: SDS−PAGE analysis of CNBr-treated cyt *c*'s: untreated (a) and CNBr-treated (b) unlabeled cyt *c*, untreated (c) and CNBr-treated (d) modified cyt *c* of the former eluted fraction of the two single-modified cyt *c*'s, and untreated (e) and CNBr-treated (f) modified cyt *c* of the latter eluted fraction.

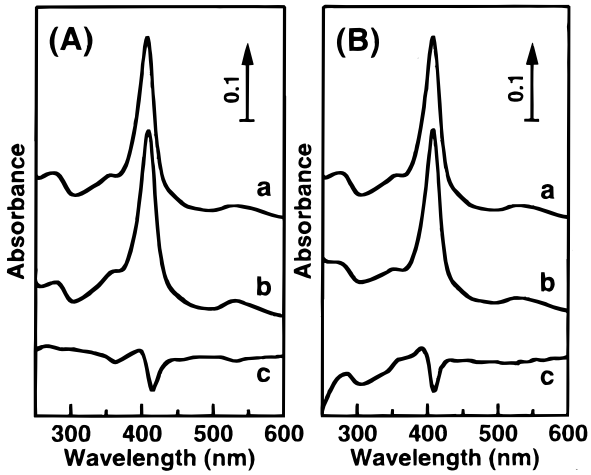


FIGURE 2: Absorption spectra in the 250−600 nm region for ferric unlabeled and NBz-Met65 cyt *c*'s: (A) NBz-Met65 (a) and unlabeled (b) cyt *c* and their difference spectrum (spectrum a − spectrum b) (c); (B) NBz-Met65 cyt *c* before (a) and after (b) irradiation of 100 shots of 308 nm laser pulses and their difference spectrum (spectrum a − spectrum b) multiplied by 3.7 (c). Concentrations of the samples: NBz-Met65 cyt *c*, 10 μM (a); unlabeled cyt *c*, 2.5 μM (Ab); NBz-Met65 cyt *c* after irradiation, 8 μM (Bb). Spectra Aa, Ba, and Bb are multiplied with factors of 0.25, 0.25, and 0.31, respectively, to adjust the intensity of the Soret absorption band. Tris-HCl buffer (50 mM), pH 7.4, was used.

Spectroscopic Characterization of NBz-Met65 Cyt c. The absorption spectra of unlabeled and NBz-Met65 cyt *c*'s and their difference spectrum are shown in Figure 2A. The Soret

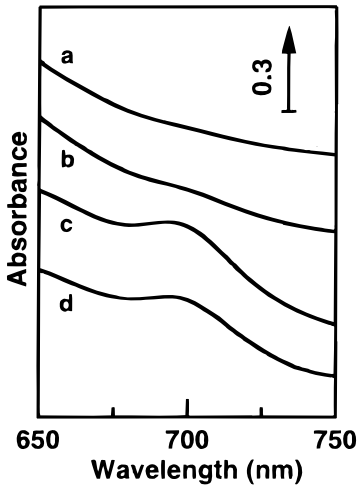


FIGURE 3: Absorption spectra in the 650−750 nm region for ferric unlabeled and NBz-Met65 cyt *c*'s: spectra before (a) and after (b) irradiation of 100 shots of 308 nm laser pulses on NBz-Met65 cyt *c* and the spectrum of unlabeled cyt *c* (c). The difference spectrum (d) represents spectrum b − spectrum a multiplied by 5. The concentration of cyt *c* was 800 μM. Other conditions were the same as those in Figure 2.

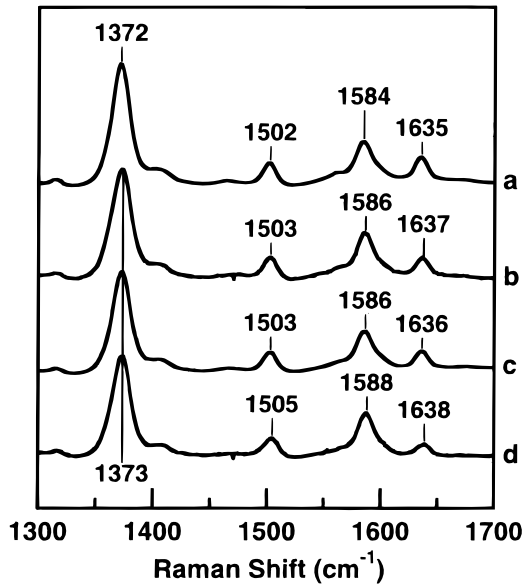


FIGURE 4: RR spectra in the 1300−1700 cm^{-1} region for ferric unlabeled (a, c) and NBz-Met65 (b, d) cyt *c*'s in the absence (a, b) and presence (c, d) of 2 M GdnHCl. Experimental conditions: slit height, 10 mm; slit width, 200 μm; excitation wavelength, 406.7 nm; laser power, 10 mW (at the sample point); sample, 20 μM in 50 mM Tris-HCl buffer, pH 7.4; room temperature.

absorption band at 409 nm of ferric cyt *c* shifted slightly to a shorter wavelength by the modification. The direction of the wavelength shift for the Soret absorption peak by the modification was the same as that observed for ferric unlabeled cyt *c* when it was unfolded by addition of GdnHCl (*42*). The intensity increase around 270 nm for the NBz-Met65 cyt *c* is attributed to incorporation of the *o*-nitrobenzyl derivative of methionine into the protein, since the absorption spectrum of the *N*-acetylmethionine *o*-nitrobenzylsulfonium chloride solution showed an absorption peak at 270 nm.

The band at 695 nm, which is related to the methionine sulfur ligation to the heme iron (*43*), disappeared for the NBz-Met65 cyt *c* (Figure 3, curve a), showing that Met80 was released from the heme iron by the modification. It is interesting that *o*-nitrobenzyl modification of Met65 for cyt *c* would cause release of Met80 from the heme iron, probably due to perturbation of the protein structure. Although Met80 was released from the heme iron, the maximum wavelength of the Soret absorption band of NBz-Met65 cyt *c* shifted less than 1 nm from that of unlabeled cyt *c* (Figure 2A), while that of ferric cyt *c* has been shown to shift by 4 nm to a shorter wavelength by unfolding the protein (*42*). These results indicate that the environment of the heme would significantly affect the maximum wavelength of the Soret absorption band of cyt *c*.

RR spectroscopy is a useful technique to obtain information on the spin and coordination states of the heme (*44*). For example, RR spectroscopy has been used to identify the heme ligand coordination state of cyt *c* for various unfolded conditions (*7*, *13*−*15*). The RR spectra of unlabeled and NBz-Met65 cyt *c*'s are shown in Figure 4. The spectra exhibited an oxidation state marker band ν_4 at 1372−1373 cm^{-1}, typical of a six-coordinate Fe(III) heme. The ν_2 and ν_3 modes, which primarily contain the $C_\beta C_\beta$ and $C_\alpha C_m$ stretching vibrations (*44*), are very sensitive to the heme ligands and spin state. The ν_2 and ν_3 bands of unlabeled cyt *c* were observed at 1584 and 1502 cm^{-1}, respectively, whereas they were detected at 1586 and 1503 cm^{-1}, respectively, for the NBz-Met65 cyt *c*, typical of a six-coordinate low-spin species. The 695 nm absorption band diminished in the absorption spectrum of NBz-Met65 cyt *c* (Figure 3, curve a), indicating that Met80 was uncoordinated from the heme iron. These results suggest that Met80 is replaced with a non-native histidine ligand, forming a bis-histidine coordinated form, for the NBz-Met65 cyt *c*. His33 has been implicated by mutant studies to be the non-native histidine ligand of unfolded cyt *c* by addition of GdnHCl at neutral pH (*3*). Similarly, His33 could be the non-native histidine ligand which coordinated to the heme iron of NBz-Met65 cyt *c*.

Since the far-UV CD spectra of proteins are related to the secondary and tertiary structures, we compared the CD spectra between unlabeled and NBz-Met65 cyt *c*'s (Figure 5A). The change in the CD spectra indicated that cyt *c* was structurally perturbed by the modification.

Stability of NBz-Met65 Cyt c against GdnHCl Denaturation. Small structural perturbation of cyt *c* has been mentioned by NMR studies on carboxymethylation of Met65 (*31*), whereas chemical modification of Met80 with iodoacetic acid has been shown to decrease stability of the protein (*32*). To investigate the influence of *o*-nitrobenzyl modification of Met65 on protein stability of cyt *c*, we measured the 222 nm CD ellipticity at various GdnHCl concentrations (Figure 6). The obtained values were fitted by assuming a two-state model with an equilibrium between the native and denatured states (*45*), and the fitted lines are overlapped in Figure 6. The C_m value, which is the transition midpoint concentration of GdnHCl for unfolding the protein, is frequently used as a probe for protein stability (*45*). The C_m value of unlabeled cyt *c* was 2.8 M in 50 mM Tris-HCl buffer, pH 7.4, at 10 °C, while that of NBz-Met65 cyt *c* decreased to 1.9 M under the same condition. The decrease in the C_m value of NBz-Met65 cyt *c* demonstrated destabilization of the protein by *o*-nitrobenzyl modification. Met65

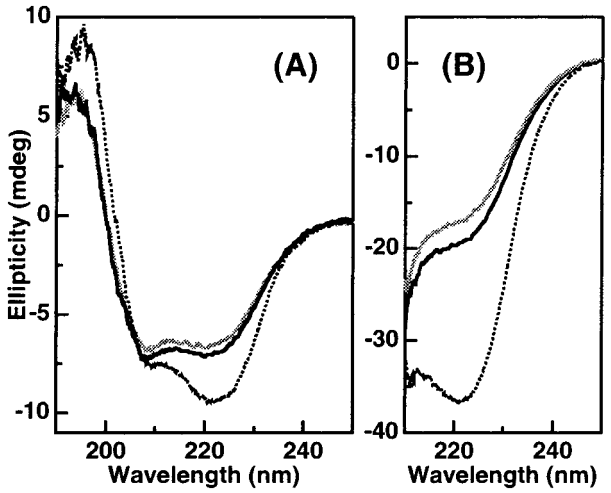


FIGURE 5: CD spectra of ferric unlabeled cyt *c* (dotted line) and NBz-Met65 cyt *c* before (gray line) and after (full line) irradiation of 100 shots of 308 nm laser pulses (A) in the absence and (B) in the presence of 2 M GdnHCl. Cyt *c* (6.5 μM) was in 20 mM sodium phosphate buffer, pH 7.2, at 10 °C.

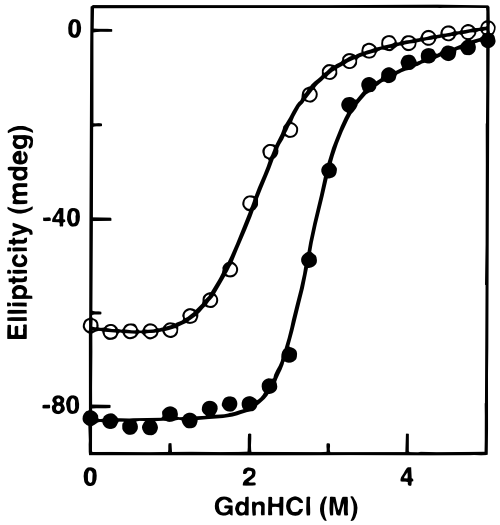


FIGURE 6: GdnHCl unfolding transitions for ferric unlabeled and NBz-Met65 cyt *c*'s. CD ellipticities at 222 nm vs GdnHCl concentration are plotted for unlabeled (●) and NBz-Met65 (O) cyt *c*'s. Fitted lines according to the published method (*45*) are also shown. Cyt *c* (36 μM) was in 50 mM Tris-HCl buffer, pH 7.4, at 10 °C.

is located at the 60s' helix which is tightly packed with the N-terminal helix, and the sulfur atom of Met65 is pointing toward the N-terminal helix. Changes in the packing of the N- and C-terminal domains of cyt *c* by site-directed mutagenesis have been reported to destabilize the protein (*46*). Therefore, *o*-nitrobenzyl modification of Met65 for cyt *c* would affect the molecular packing between the 60s' and N-terminal helices and thus may perturb the protein structure and decrease its stability.

Irradiation of UV Light on NBz-Met65 Cyt c. The absorption spectra of NBz-Met65 cyt *c* before and after irradiation of 100 shots of laser pulses at 308 nm and their difference spectrum are shown in Figure 2B. The peak position of the Soret band of ferric cyt *c* which shifted to a shorter wavelength by *o*-nitrobenzyl modification of Met65 shifted backward to a longer wavelength with irradiation of a UV light on the NBz-Met65 cyt *c*. The pattern of the difference absorption spectrum of NBz-Met65 cyt *c* before and after irradiation of a UV light was similar to that between NBz-Met65 and unlabeled cyt *c*'s (Figure 2), suggesting that cyt *c* recovered its native heme coordination structure with the UV irradiation, due to recovery of unlabeled Met65 by releasing 2-nitrosobenzaldehyde. Reaction of the *o*-nitrobenzyl derivative for the NBz-Met65 cyt *c* by the UV irradiation was verified by the decrease in the 270 nm absorption band, whereas formation of 2-nitrosobenzaldehyde by the irradiation was confirmed by the absorption increase around 320 nm (Figure 2B, curve c), since 2-nitrosobenzaldehyde has a strong absorption band around 320 nm. The amount of the heme which recovered its heme environment by irradiation of 100 shots of UV pulses was estimated to be about 27% from the intensity of the difference pattern of the Soret absorption band (Figure 2). The 695 nm absorption band also appeared with irradiation of the UV light on the NBz-Met65 cyt *c*, demonstrating recoordination of Met80 to the heme iron (Figure 3, curve a → b). The amount of the heme which recovered its methionine coordination by irradiation of 100 shots of UV pulses was estimated to be about 20% from the intensity increase of the 695 nm absorption band.

The CD spectra of the far-UV region before and after irradiation of 100 shots of laser pulses at 308 nm on the NBz-Met65 cyt *c* are also shown in Figure 5A. Negative peaks at 208 and 222 nm in the CD spectra are characteristic of α-helices. However, these peaks are also influenced by the tertiary structure with contribution from the side chains. The intensity of the negative peak around 222 nm in the CD spectra of NBz-Met65 cyt *c* increased by the UV irradiation, demonstrating recovery of the native protein structure by conversion of NBz-Met65 to unlabeled methionine. For example, the 220 nm CD ellipticity regained 19% of its modification-reduced intensity with irradiation of UV pulses. The estimated proportion which regained the native conformation with irradiation of UV pluses on the NBz-Met65 cyt *c* corresponded relatively well between absorbance and CD measurements, suggesting that the NBz-Met65 cyt *c* converted to the native conformation with the UV irradiation.

Time-Resolved Absorption Changes by UV Irradiation on NBz-Met65 Cyt c. As discussed above, the structurally perturbed NBz-Met65 cyt *c* restored to its native structure and recovered its methionine heme coordination by the UV irradiation. Therefore, time-resolved absorption changes of NBz-Met65 cyt *c* initiated by irradiation of a single laser pulse at 308 nm were investigated. The time-resolved absorption measurements were performed in the presence of 2 M GdnHCl, since a significant amount of NBz-Met65 cyt *c* was unfolded in 2 M GdnHCl, whereas unlabeled cyt *c* was almost folded under this condition (see Figure 6). The RR spectra of unlabeled and NBz-Met65 cyt *c*'s in 2 M GdnHCl were both characteristic of six-coordinate low-spin species (Figure 4). The 695 nm absorption band was undetectable for the NBz-Met65 cyt *c*, while that of unlabeled cyt *c* decreased its intensity by 35% in the presence of 2 M GdnHCl (data not shown), indicating release of methionine ligation from the heme iron. The NBz-Met65 cyt *c* in 2 M GdnHCl should therefore be a bis-histidine coordinated species, while unlabeled cyt *c* in the same condition should be a mixture of bis-histidine and histidine−methionine

coordinated species. UV irradiation on the NBz-Met65 cyt *c* in the presence of 2 M GdnHCl would represent an equilibrium shift from a mixture of folded and unfolded bis-histidine coordinated species to a mixture of folded bis-histidine and histidine−methionine coordinated species (Figure 8).

The CD spectra before and after UV irradiation on the NBz-Met65 cyt *c* in the presence of 2 M GdnHCl are shown in Figure 5B, together with the spectrum of the unlabeled protein in 2 M GdnHCl. The CD spectrum below 210 nm was not obtainable, due to strong absorbance by GdnHCl. Recovery of the native structure by the UV irradiation in the presence of 2 M GdnHCl was estimated from the ellipticity at 222 nm to be about 14%. The heme conformation was estimated to recover about 13% with irradiation of UV pulses in the presence of 2 M GdnHCl, as judged from the change in the Soret absorption (data not shown). Restoration to its methionine-coordinated heme structure by the UV irradiation in the presence of 2 M GdnHCl was estimated to be about 11% from the intensity increase of the 695 nm absorption band (data not shown). From these Soret absorption and CD measurements, similar proportions were obtained for recovery of the unlabeled protein with the UV irradiation on the NBz-Met65 cyt *c* in the presence of 2 M GdnHCl, which demonstrated recovery of unlabeled cyt *c* by irradiation of UV pulses even in 2 M GdnHCl.

The absorbance changes at 401 and 418.5 nm after irradiation of a 308 nm pulse light on the NBz-Met65 cyt *c* in the presence of 2 M GdnHCl are shown in Figure 7, together with the least-squares exponential fits. A single exponential fit was successful for the fast phase (Figure 7A), while the fit for the later reactions was only successful with a double exponential fit (Figure 7B). An intermediate was detected as a Soret absorbance change with a rate constant of 21 000 ± 4000 s^{-1} by irradiation of a 308 nm pulse on the NBz-Met65 cyt *c* in the presence of 2 M GdnHCl (Figure 7A). This fast phase was missing for irradiation of the same UV pulse on the unlabeled cyt *c* in the presence of 2 M GdnHCl or on the NBz-Met65 cyt *c* in the absence of it (data not shown). The wavelength dependence of the relative intensity of the absorbance change for this fast phase is shown in the inset of Figure 7A. The Soret maximum wavelength of the intermediate was estimated to be slightly longer (about 1 nm) than that of NBz-Met65 cyt *c*. This intermediate decayed with a rate constant of 90 ± 15 s^{-1}, followed by another phase with a rate constant of 13 ± 3 s^{-1} (Figure 7B). The amount of the reacted species by irradiation of a single pulse was estimated to be about 5% from the final absorption changes in Figure 7B. Detection of the ~10 ms phase suggested that perturbation around the heme site would occur during formation of α-helices, since formation of α-helices have been observed at this time region (*23*). Formation of the native histidine−methionine coordinated species from the bis-histidine coordinated species is reported by RR spectroscopy to pass though another state, which was suggested to be the histidine−water coordinated form (*13*). The time scale of ~80 ms was similar to the time scales of the ligand exchange processes, and this phase may correspond to the histidine−water and/or histidine−methionine ligand exchange processes, although the rate constants differed a little from the reported values due to the differences in the measuring conditions (*10*, *13*−*15*). However, a similar

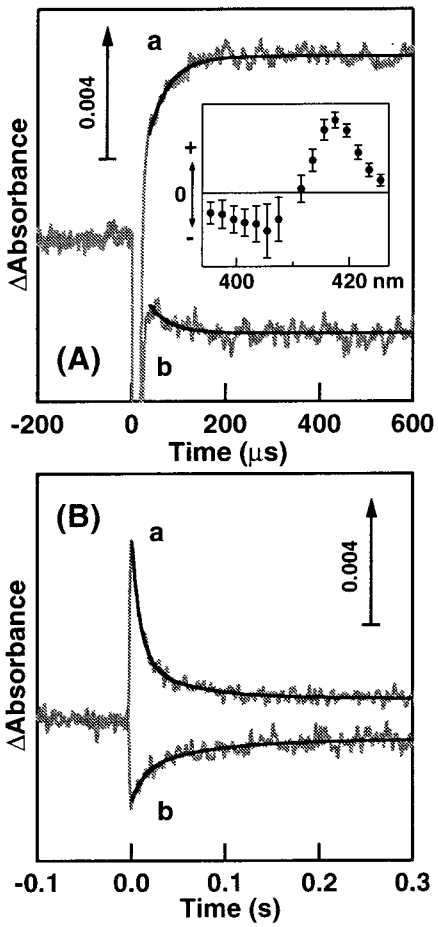


FIGURE 7: Time-resolved absorbance changes at 418.5 (a) and 401 nm (b) by irradiation of a single 308 nm pulse on ferric NBz-Met65 cyt *c* in the presence of 2 M GdnHCl: (A) −200 to 600 μs and (B) −0.1 to 0.3 s. The wavelength dependence of the relative intensity of the absorbance change for the fast phase is shown in the inset. The laser power was about 12 mJ per pulse per cm^2. Cyt *c* (8 μM) was in 50 mM Tris-HCl buffer, pH 7.4, at 10 °C.

intermediate was also observed for Met80 *o*-nitrobenzyl-modified cyt *c* which decayed with a triphasic dependence (*41*), and the slowest phase detected in this study for the NBz-Met65 cyt *c* might be an overlap of two phases.

The kinetics of the early intermediate was not affected by introducing CO or removing O_2, which neglects the possibility of formation of the ferrous cyt *c* species by photoreduction. The submillisecond protein folding dynamics of cyt *c* in ferric and ferrous states have been studied by time-resolved tryptophan fluorescence (*16*, *26*) and CD (*23*) methods. According to the stopped-flow CD measurements of ferric cyt *c* folding, about 44% of the α-helical signal of the protein formed within the dead time of the measurement, indicating that a significant amount of the secondary structure was formed within 4 ms (*19*). More recently, time-resolved CD measurements of reduced cyt *c* folding showed almost no change in the secondary structure of the protein between 16 μs and 1 ms (*23*). However, tryptophan fluorescence exhibited an exponential phase with a time constant of about 50 μs for ferric cyt *c* folding, indicating that the protein collapse during protein folding occurred within 100 μs (*26*) (Figure 8). Since the protein collapse occurred earlier than formation of the α-helices, a loosely packed intermediate should be formed at the early stage of cyt *c* folding. We

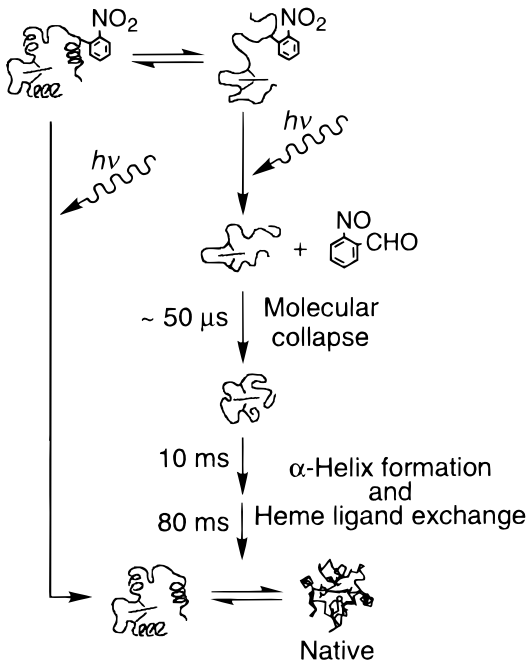


FIGURE 8: Tentative folding process of cyt *c* by UV light irradiation on NBz-Met65 cyt *c* in the presence of 2 M GdnHCl.

have discussed above that the maximum wavelength of the Soret absorption band would be significantly affected by the environment of the heme. Since the time scale for formation of the intermediate species detected in this study corresponded well with that for the protein collapse observed by tryptophan fluorescence (about 50 μs) (*26*) and the protein collapse could affect the structure of the heme site, the early intermediate may be attributed to perturbation in the bis-histidine coordination structure caused by the protein collapse (Figure 8). Observation of the fast 50 μs phase under constant pressure and denaturant concentration suggests that the fast phase observed by tryptophan fluorescence (*26*) is not caused by the change in the solution state due to mixing the two solutions at high pressure. Finally, we suggest that the *o*-nitrobenzyl modification in this study would be a useful method for studying protein dynamics of other proteins at their early folding stages.

In summary, Met65 cyt *c* was chemically modified with *o*-nitrobenzyl bromide to form a photocleavable *o*-nitrobenzyl derivative of Met65, and the folding character of cyt *c* was studied with the use of this modified protein and subsequent UV light irradiation. Met80 was released from the heme iron, and the protein structure of cyt *c* was perturbed by the modification. The native folding structure of cyt *c* recovered by converting NBz-Met65 to unlabeled methionine with irradiation of a UV light. We detected a fast phase as a change in Soret absorption with a rate constant of 21 000 $\pm$ 4000 s^{-1} during refolding of cyt *c* initiated by irradiation of a 308 nm pulse on the NBz-Met65 cyt *c* in the presence of 2 M GdnHCl. The rate constant for the fast phase corresponded well with that observed by the tryptophan fluorescence study (*26*).

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REFERENCES

1. Bushnell, G. W., Louie, G. V., and Brayer, G. D. (1990) *J. Mol. Biol. 214*, 585−595.
2. Banci, L., Bertini, I., Gray, H. B., Luchinat, C., Reddig, T., Rosato, A., and Turano, P. (1997) *Biochemistry 36*, 9867−9877.
3. Colón, W., Wakem, L. P., Sherman, F., and Roder, H. (1997) *Biochemistry 36*, 12535−12541.
4. Muthukrishnan, K., and Nall, B. T. (1991) *Biochemistry 30*, 4706−4710.
5. Babul, J., and Stellwagen, E. (1972) *Biochemistry 11*, 1195−1200.
6. Dyson, H. J., and Beattie, J. K. (1982) *J. Biol. Chem. 257*, 2267−2273.
7. Jordan, T., Eads, J. C., and Spiro, T. G. (1995) *Protein Sci. 4*, 716−728.
8. Hamada, D., Kuroda, Y., Kataoka, M., Aimoto, S., Yoshimura, T., and Goto, Y. (1996) *J. Mol. Biol. 256*, 172−186.
9. Wallace, C. J. A., and Clark-Lewis, I. (1992) *J. Biol. Chem. 267*, 3852−3861.
10. Brems, D. N., and Stellwagen, E. (1983) *J. Biol. Chem. 258*, 3655−3660.
11. Elöve, G. A., Bhuyan, A. K., and Roder, H. (1994) *Biochemistry 33*, 6925−6935.
12. Pierce, M. M., and Nall, B. T. (1997) *Protein Sci. 6*, 618−627.
13. Takahashi, S., Yeh, S.-R., Das, T. K., Chan, C.-K., Gottfried, D. S., and Rousseau, D. L. (1997) *Nat. Struct. Biol. 4*, 44−50.
14. Yeh, S.-R., Takahashi, S., Fan, B., and Rousseau, D. L. (1997) *Nat. Struct. Biol. 4*, 51−56.
15. Yeh, S.-R., Han, S., and Rousseau, D. L. (1998) *Acc. Chem. Res. 31*, 727−736.
16. Chan, C.-K., Hu, Y., Takahashi, S., Rousseau, D. L., Eaton, W. A., and Hofrichter, J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1779−1784.
17. Myer, Y. P., Pande, A., and Saturno, A. F. (1981) *J. Biol. Chem. 256*, 1576−1581.
18. Pryse, K. M., Bruckman, T. G., Maxfield, B. W., and Elson, E. L. (1992) *Biochemistry 31*, 5127−5136.
19. Elöve, G. A., Chaffotte, A. F., Roder, H., and Goldberg, M. E. (1992) *Biochemistry 31*, 6876−6883.
20. Sosnick, T. R., Mayne, L., Hiller, R., and Englander, S. W. (1994) *Nat. Struct. Biol. 1*, 149−156.
21. Sauder, J. M., and Roder, H. (1998) *Folding Des. 3*, 293−301.
22. Telford, J. R., Wittung-Stafshede, P., Gray, H. B., and Winkler, J. R. (1998) *Acc. Chem. Res. 31*, 755−763.
23. Chen, E., Wittung-Stafshede, P., and Kliger, D. S. (1999) *J. Am. Chem. Soc. 121*, 3811−3817.
24. Telford, J. R., Tezcan, F. A., Gray, H. B., and Winkler, J. R. (1999) *Biochemistry 38*, 1944−1949.
25. Jones, C. M., Henry, E. R., Hu, Y., Chan, C.-K., Luck, S. D., Bhuyan, A., Roder, H., Hofrichter, J., and Eaton, W. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11860−11864.
26. Shastry, M. C. R., and Roder, H. (1998) *Nat. Struct. Biol. 5*, 385−392.
27. Shastry, M. C. R., Sauder, J. M., and Roder, H. (1998) *Acc. Chem. Res. 31*, 717−725.
28. Stellwagen, E. (1968) *Biochemistry 7*, 2496−2501.
29. Osheroff, N., Brautigan, D. L., and Margoliash, E. (1980) *J. Biol. Chem. 255*, 8245−8251.
30. Theodorakis, J. L., Garber, E. A. E., McCracken, J., Peisach, J., Schejter, A., and Margoliash, E. (1995) *Biochim. Biophys. Acta 1252*, 103−113.
31. Boswell, A. P., Moore, G. R., Williams, R. J. P., Wallace, C. J. A., Boon, P. J., Nivard, R. J. F., and Tesser, G. I. (1981) *Biochem. J. 193*, 493−502.
32. de Jongh, H. H. J., Goormaghtigh, E., and Ruysschaert, J.-M. (1995) *Biochemistry 34*, 172−179.

33. Yang, Y., and Zhou, H.-M. (1998) *Biochim. Biophys. Acta 1388*, 190−198.
34. Yang, Y., Park, Y.-D., Yu, T. W., and Zhou, H. M. (1999) *Biochem. Biophys. Res. Commun. 259*, 450−454.
35. Lu, H. S. M., Volk, M., Kholodenko, Y., Gooding, E., Hochstrasser, R. M., and DeGrado, W. F. (1997) *J. Am. Chem. Soc. 119*, 7173−7180.
36. McCray, J. A., and Trentham, D. R. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 239−270.
37. Ramesh, D., Wieboldt, R., Niu, L., Carpenter, B. K., and Hess, G. P. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11074−11078.
38. Hess, G. P., and Grewer, C. (1998) *Methods Enzymol. 291*, 443−473.
39. Pollitt, S. K., and Schultz, P. G. (1998) *Angew. Chem., Int. Ed. Engl. 37*, 2104−2107.
40. Corradin, G., and Harbury, H. A. (1970) *Biochim. Biophys. Acta 221*, 489−496.
41. Okuno, T., Hirota, S., and Yamauchi, O. (2000) *Chem. Lett.* 290−291.
42. Stellwagen, E. (1968) *Biochemistry 7*, 2893−2898.
43. Schejter, A., and George, P. (1964) *Biochemistry 3*, 1045−1049.
44. Hu, S., Morris, I. K., Singh, J. P., Smith, K. M., and Spiro, T. G. (1993) *J. Am. Chem. Soc. 115*, 12446−12458.
45. Santoro, M. M., and Bolen, D. W. (1988) *Biochemistry 27*, 8063−8068.
46. Colón, W., Elöve, G. A., Wakem, L. P., Sherman, F., and Roder, H. (1996) *Biochemistry 35*, 5538−5549.

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