# Folding Properties of Cytochrome *c* Studied by Photocleavable *o*-Nitrobenzyl Modification of Methionine 65 and 80

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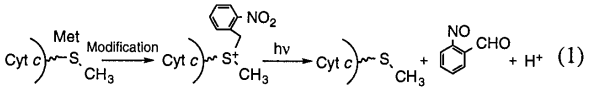
Folding properties of cytochrome *c* (cyt *c*) were studied by specific *o*-nitrobenzyl modification of its methionines and subsequent UV light irradiation. Absorption changes with time scales of about 50 μs, 10 ms, 30–100 ms, and 250–500 ms were observed during folding of cyt *c*.

Cyt *c* is a heme protein with three α-helices. Its molecular recognition and structural properties have been studied by chemical modification,[1] where modification of Met65 and Met80 have caused structural perturbation.[1c,d] Cyt *c* has been used as a model to study the folding character of proteins.[2] However, folding properties of proteins for time scales faster than milliseconds are not well understood due to limitation of useful methods. A photocleavable disulfide bond has been introduced into a model peptide, and reformation of its secondary structure has been observed by irradiation of a UV light.[3] Photoreactive *o*-nitrobenzyl-substituted amino groups have been shown to produce amino groups with irradiation of a UV light.[4] Similar *o*-nitrobenzyl groups have been introduced into proteins.[5] In this study, we made two single-methionine-modified cyt *c*'s by introducing a photocleavable *o*-nitrobenzyl group at Met65 or Met80 and irradiated a UV light on the modified proteins to study the structural and folding properties of cyt *c*.




Specific modification of the methionine thioether group of cyt *c* with iodoacetic acid has been performed at pH 1.5 to protonate other reactive residues,[1d] since many lysines would be modified at neutral pH. Chemical modification of methionines of cyt *c* to their *o*-nitrobenzyl derivatives was performed by mixing horse heart cyt *c* (90 mg) in 50 mM glycine-HCl 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 in the dark. Urea and $(NH_4)_2SO_4$ were added to the modified protein solution to final concentrations of 3 and 2 M, respectively. The modified proteins were separated with a Phenyl-Toyopearl column (Figure 1). Native cyt *c* (Fraction I) 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 the modified cyt *c*'s, 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 was performed. The first two fractions (Fractions II and III), 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 at 270 nm by the modification with the intensity of the 270-nm absorption band of the *N*-acetylmethionine *o*-nitrobenzylsulfonium chloride solution.

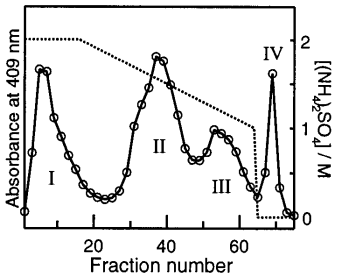



**Figure 1.** Elution curve of *o*-nitrobenzyl modified horse heart cyt *c*'s. Absorbance at 409 nm. The dotted line represents the concentration of $(NH_4)_2SO_4$.

CNBr is well known to preferentially react with the side chain of Met65 of cyt *c* and cleave the protein.[6] Native and the two single-methionine-modified cyt *c* solutions were subjected to the CNBr reaction and subsequent SDS-polyacrylamide gel electrophoresis analysis. CNBr-treated native cyt *c* exhibited a smaller molecular weight than native cyt *c* due to cleavage at Met65 by CNBr, while the amount of the cleaved protein by CNBr decreased significantly for the modified cyt *c* of Fraction III. These results indicated that the modified methionine for the protein of Fraction III was Met65. Since the modified cyt *c* of Fraction II contained one *o*-nitrobenzyl methionine and reacted with CNBr, the protein of Fraction II should be modified at Met80. The most hydrophobic fraction (Fraction IV) which started to elute with the lowest $(NH_4)_2SO_4$ concentration contained both *o*-nitrobenzylmethionine derivatives according to the intensity increase of its 270-nm absorption band.

To investigate the protein stability of modified cyt *c*'s, we measured the circular dichroism (CD) ellipticity at 222 nm at various guanidine hydrochloride (GdnHCl) concentrations. The data obtained were fitted by assuming a two-state model with an equilibrium between the native and denatured states.[7] 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.[7] The $C_m$ value of native cyt *c* was 2.8 M in 50 mM Tris-HCl buffer, pH 7.4, at 10 ˚C, while the $C_m$ values of Met65 and Met80 modified cyt *c*'s decreased to 1.9 and 2.5 M, respectively, under the same condition. The decrease in the $C_m$ value for modified cyt *c*'s demonstrated destabilization of the protein by the modification, and the Met65 modified protein was less stable than the Met80 modified protein. The difference in protein stability corresponded well with the result that Met65 modified cyt *c* eluted with lower $(NH_4)_2SO_4$ concentration than Met80 modified cyt *c* in the presence of 3 M urea (Figure 1).

The 695-nm absorption band disappeared for modified cyt

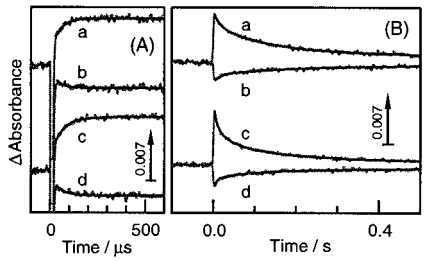



**Figure 2.** Time-resolved absorbance changes at 418.5 (a,c) and 401 (b,d) nm by irradiation of a 308-nm pulse on ferric *o*-nitrobenzyl modified cyt *c* at Met80 (a,b) or Met65 (c,d) in the presence of 2 M GdnHCl; (A) -100–600 μs and (B) -0.1–0.5 s. Cyt *c* (8 μM) in 50 mM Tris-HCl buffer, pH 7.4, at 10 °C. Pulse intensity was 12 mJ/cm$^2$.

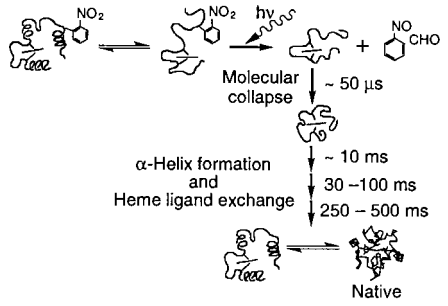



**Figure 3.** Tentative model for folding of cyt *c* by UV light irradiation on modified cyt *c* in the presence of 2 M GdnHCl.

*c*'s, showing that Met80 was released from the heme iron. Their resonance Raman (RR) spectra were typical of six-coordinate low-spin species, suggesting that modified cyt *c*'s were bis-histidine coordinated. The Soret maximum wavelength of cyt *c* shifted less than 1 nm by the modification, although Met80 was released from the heme iron, while it shifted by 4 nm to a shorter wavelength by unfolding the protein. These results indicated that the environment of the heme would significantly affect the Soret absorption band of cyt *c*. Cyt *c* started to refold by reconversion of the *o*-nitrobenzyl methionine to its native form by irradiation of a 308-nm pulse. Time-resolved absorption measurements were performed in the presence of 2 M GdnHCl, since a significant amount of modified cyt *c* was unfolded, whereas native cyt *c* was folded with 2 M GdnHCl. UV pulse irradiation on modified cyt *c* in the presence of 2 M GdnHCl would cause an equilibrium shift from a mixture of folded and unfolded bis-histidine coordinated species to a mixture of folded bis-histidine coordinated and histidine-methionine coordinated species. The intensity changes in the absorption in the Soret region by irradiation of the UV pulse on modified cyt *c* were prominent at 401 and 418.5 nm, which represented a wavelength shift of the Soret band. Their time dependence are shown in Figure 2. A single exponential fit was successful for the fast phase (Figure 2A), while the later reactions were fitted with triple exponential curves (Figure 2B). Intermediates were detected as Soret absorbance changes with rate constants of 22000 ± 2000 and 20000 ± 3000 $s^{-1}$ for Met80 and Met65 modified cyt *c*'s, respectively. The intermediates decayed with rate constants of 93 ± 10 and 110 ± 10 $s^{-1}$, followed by two more phases (rate constants; 10 ± 3 and 2 ± 1 $s^{-1}$ and 30 ± 5 and 4 ± 2 $s^{-1}$ for Met80 and Met65 modified proteins, respectively). No significant absorption change was observed for irradiation of the pulse on the modified proteins without GdnHCl or on native cyt *c* with 2 M GdnHCl. The kinetics of the intermediates were not affected by introducing CO or removing $O_2$, which neglects the possibility of photoreduction of the heme. Interestingly, the early intermediate was observed for both modified cyt *c*'s with similar time constants, suggesting it is a species with a common character. Since the time scale for the formation of the early intermediate detected in this study corresponded well with that of the collapse of the polypeptide chain observed by tryptophan fluorescence (about 50 μs),[2b] the intermediate may be attributed to perturbation in the bis-histidine coordination structure caused by the molecular collapse (Figure 3). The absorbance changes were a little larger for the Met65 modified protein than for the Met80 modified protein, probably due to the more unfolded character for the former protein. Detection of the ~10 ms phase suggested that perturbation around the heme site would occur by formation of α-helices, since their formation has been observed at this time region.[2c] The other slower phases might correspond to the heme ligand exchange processes, which have been previously observed by RR spectroscopy.[2d,e] Finally, we infer that site specific photoreactive *o*-nitrobenzyl modification of amino acid residues of proteins would be a very useful method to study the stability and folding characters of proteins.

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