# Ruthenium-Mediated Protein Cross-Linking and Stabilization

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General strategies for stabilizing the three-dimensional structures of proteins will expand their potential applications in biotechnology and will aid in the design of novel proteins. Here we report a dramatic increase in the conformational and thermal stability of a protein obtained by cross-linking an engineered metal-binding site with a substitution-inert metal complex. Cross-linking two histidines (His39 and His58) on opposite strands of a $\beta$-sheet with $Ru^{II}(bpy)_2$ (where bpy is 2,2′-bipyridine) increases the unfolding free energy ($\Delta G_u°$) of *Saccharomyces cerevisiae* iso-1-cytochrome *c* by 5.5 kcal mol$^{-1}$ at 25 °C (to 9.5 kcal mol$^{-1}$). The melting temperature of the cross-linked protein increases by 23.2 °C (to 72.8 °C). This cross-linking is accompanied by a minimal change in the cytochrome *c* $Fe^{III/II}$ reduction potential.

Two site-directed mutants of iso-1-cytochrome *c* were constructed, each having a dihistidine metal-chelating site on the protein surface.[1] Introduction of a histidine at position 58 creates a chelating site with the native His39 located across a short segment of antiparallel $\beta$-sheet (designated $H_{39}H_{58}$) (Figure 1). The second variant has the exposed His39 replaced with glutamine and a chelating site in which the two histidines are separated by a single turn (His-$X_3$-His) of the N-terminal $\alpha$-helix ($H_4H_8$). Both variants contain a cysteine-to-serine mutation at 102 to prevent oxidative dimerization ($S_{102}$). The mutants were expressed in functional form in *S. cerevisiae* and were strongly retained during immobilized metal-affinity chromatography (IMAC), indicating that the surface dihistidine sites chelate $Cu^{II}$.[1]

Reaction of the cytochromes *c* with excess $Ru^{II}(bpy)_2CO_3·H_2O$ under an inert atmosphere was monitored spectroscopically and by IMAC.[3,4] Multiply ruthenated proteins were separated from those modified at a single site by cation exchange chromatography. The protein fraction corresponding to modification with a single $Ru^{II}(bpy)_2$ was further fractionated by IMAC to obtain the pure, $Ru^{II}$-cross-linked protein.[4] That both histidines of the chelating sites were coordinated to $Ru^{II}(bpy)_2$ was confirmed by treatment with diethyl pyrocarbonate (DEPC)[5] and amino acid sequencing of the $Ru^{II}$-containing peptide product of tryptic digestion.[6] Further evidence comes from the appearance in the modified proteins of the 670-nm emission band observed in the model compound $Ru^{II}(bpy)_2(imidazole)_2$.[4]

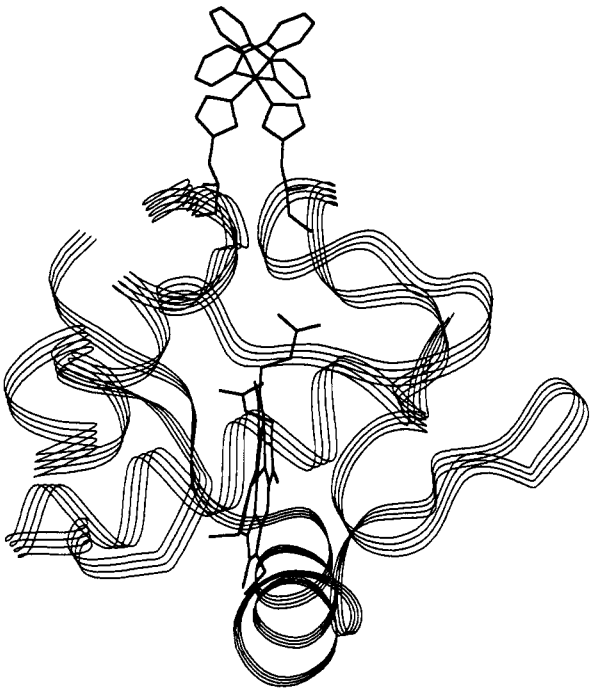

**Figure 1.** Energy-minimized model of $Ru^{II}(bpy)_2$-$H_{39}H_{58}$ cytochrome *c*. The calculation employed Biograf, Version 3.0. Coordinates for the $Ru^{II}$ complex were taken from the structure of $Ru^{II}(bpy)_2Cl_2$.[2] Two histidines cross-linking a $\beta$-sheet provide an appropriate geometry for metal chelation; thus the cross-link introduces minimal changes in the backbone structure upon energy minimization.

Because the cytochrome *c* reduction potential is sensitive to conformational changes that affect the heme pocket, half-wave potentials of the unmodified and modified proteins were measured by cyclic voltammetry.[7,8] The $H_{39}H_{58}$ variant exhibits a slightly higher reduction potential (0.290(5) V vs NHE, pH 7.0, 25 °C) than the $S_{102}$ single-site mutant (0.272(5) V).[8] Cross-linking His39 and His58 with $Ru^{II}$ partially restores the heme potential (0.282(5) V) to that of the $S_{102}$ cytochrome *c*. The potential of the $H_4H_8$ variant (0.254(5) V) is very close to that of its parent molecule ($Q_{39}S_{102}$ cytochrome *c*, 0.260(5) V), and $Ru^{II}$ modification has a very small effect on the reduction potential (0.257(5) V). Surface chelation of the $Ru^{II}$ complex does not appear to perturb the protein conformation near the heme pocket.

As shown in Figure 2, cross-linking with $Ru^{II}$ increases the melting temperature ($T_m$) of $H_{39}H_{58}$ cytochrome *c* by more than 23 °C, from 49.6 to 72.8 °C (a 16.5 °C increase over the $T_m$ of the wild-type protein), while the concentration of the denaturant guanidinium chloride (GdmCl), at which the protein is half unfolded, is more than doubled, to 2.27 M. The free energy of unfolding ($\Delta G_u°$) of the $Ru^{II}$-cross-linked protein is 9.5 kcal/mol at 25 °C, an increase of 5.5 kcal/mol over the unmodified protein and 4.2 kcal/mol greater than wild-type protein.[9] Such stabi-

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(2) Eggleston, D. S.; Goldsby, K. A.; Hodgson, D. J.; Meyer, T. J. *Inorg. Chem.* **1985**, *24*, 4573–4580.

(3) A 5–10 times molar excess of $Ru^{II}(bpy)_2CO_3·H_2O$ was added to a degassed 0.2 mM protein solution. The reaction was monitored periodically by measuring the absorbance $A_{292}/A_{410}$ of samples desalted by gel-filtration chromatography.[4] At a Ru:protein ratio of 1:1 ($A_{292}/A_{410} = 2/3$), the reaction was stopped by desalting on a Sephadex G-25 column equilibrated with the reaction buffer. Samples were loaded onto a Mono-S HR 16/10 column (Pharmacia), and the proteins were eluted with a 0–0.5 M NaCl gradient in 100 mM sodium phosphate buffer ($NaP_i$), pH 7.2. The major peak containing the 1:1 Ru-modified products was applied to a $Cu^{II}$-IMAC column (G6000PW (Toya Soda) or HiTrap (Pharmacia)) in 50 mM $NaP_i$, pH 7.0, 0.5 M NaCl, and 1 mM imidazole and eluted with a gradient of 1–20 mM imidazole over 60 min in the loading buffer. The first protein peak was collected, and its buffer was exchanged by gel filtration in 50 mM $NaP_i$, pH 7.0. Proteins were concentrated by ultrafiltration and stored at 4 °C. Once a histidine is coordinated to $Ru^{II}$, it becomes inaccessible to the matrix-bound metal ion in IMAC, altering retention in a predictable manner (see, for example, Arnold, F. H. *Bio/Technology* **1991**, *9*, 151–156).

(4) Durham, B. D.; Pan, L. P.; Hahm, S.; Long, J.; Millett, F. *Adv. Chem. Ser.* **1990**, *226*, 181–193.

(5) A 20 $\mu$M cytochrome *c* solution in 0.1 M $NaP_i$, pH 7.0 was treated with excess DEPC (Jackman, M. P.; Lim, M.-C.; Osvath, P.; De Silva, H.; Sykes, A. G. *Inorg. Chim. Acta* **1988**, *153*, 205–208). The $Ru^{II}$-modified metal-chelating cytochrome *c* variant reacted similarly to the $Q_{39}S_{102}$ variant, which lacks His39 and the engineered histidines.

(6) The $Ru^{II}$-containing fragment of the tryptic digest of $Ru^{II}$-cross-linked $His_{39}His_{58}$ protein was isolated and sequenced. The metal complex cross-links two tryptic peptides (39–54 and 56–72), and the expected mixture of amino acids at each cleavage cycle was observed.

(7) Electrochemical measurements were performed with a gold electrode modified with 4,4′-dipyridyldisulfide (Aldrich) and a Ag/AgCl/KCl reference electrode (Allen, P. M.; Hill, H. A. O.; Walton, N. J. *J. Electroanal. Chem.* **1984**, *178*, 69–86). A scan rate of 10 mV s$^{-1}$ was employed.

(8) Casimiro, D. R.; Richards, J. H.; Winkler, J. R.; Gray, H. B., submitted for publication.

0002-7863/93/1515-5312$04.00/0 

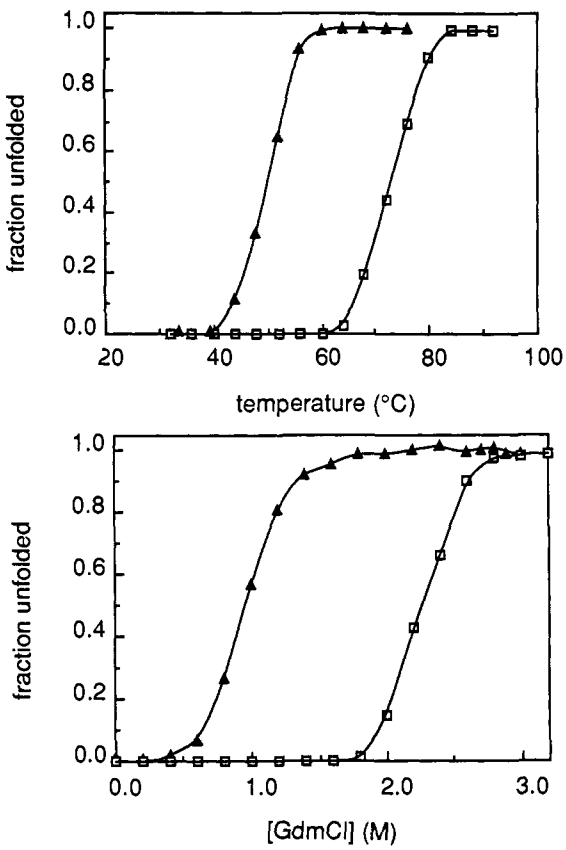


**Figure 2.** Stabilization of $H_{39}H_{58}$ cytochrome *c* by $Ru^{II}(bpy)_2$ (pH 7.0). (a) Thermal unfolding of unmodified (▲) and $Ru^{II}$-cross-linked (□) protein. (b) GdmCl-induced unfolding of unmodified (▲) and $Ru^{II}$-cross-linked (□) protein.

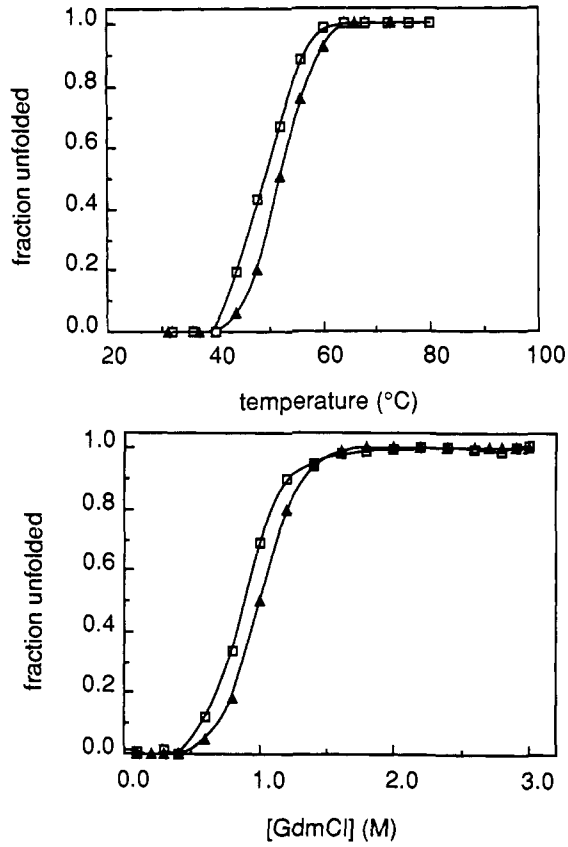


**Figure 3.** Stabilization of $H_4H_8$ cytochrome *c* by $Ru^{II}(bpy)_2$ (pH 7.0). (a) Thermal unfolding of unmodified (▲) and $Ru^{II}$-cross-linked (□) protein. (b) GdmCl-induced unfolding of unmodified (▲) and $Ru^{II}$-cross-linked (□) protein.

lization is not observed when cytochrome *c* not containing the metal-chelating site (i.e., $S_{102}$ cytochrome *c*, which has His39 but not His58) is modified with $Ru^{II}(bpy)_2$(imidazole) (data not shown).

$Ru^{II}(bpy)_2$ connects two histidine residues by simultaneously coordinating the imidazole side chains. Because the chelated metal is substitution-inert, the $Ru^{II}$ cross-link is retained when the protein unfolds and therefore resembles a disulfide bridge.[10] The stabilizing effect of $Ru^{II}$ cross-linking is comparable to that of natural disulfides: removing one of the four disulfides in native hen lysozyme (connecting residues 6 and 127) lowered $T_m$ from 77 to 53 °C,[11] while the $T_m$ of ribonuclease $T_1$ decreased from 59 to 27 °C when two of its disulfide bonds were deleted.[12]

The mechanism by which such a cross-link stabilizes the protein is widely thought to involve a reduction in the chain entropy of the denatured state and therefore depends on the size of the loop formed by the cross-link.[12,13] We therefore tested a second $Ru^{II}$ cross-link that forms a loop of only four residues in the N-terminal α-helix of cytochrome *c*. As shown in Figure 3, $H_4H_8$ cytochrome *c* is not stabilized by cross-linking with $Ru^{II}(bpy)_2$; its $T_m$ is in fact reduced slightly (by 2.0 °C), as is the resistance to denaturation by GdmCl. Although ruthenium cross-linking has been reported to stabilize helical peptides containing a His-$X_3$-His site, the values of $\Delta\Delta G_u°$ are at most only 1 kcal $mol^{-1}$.[14] Cross-linking a single turn of an α-helix would likely not confer a high degree of stabilization to a protein.

The metal-mediated β-sheet cross-link offers a high degree of stabilization comparable to naturally-occuring disulfide bridges; it also avoids many of the difficulties encountered when new disulfides are engineered into proteins. For example, engineered disulfides often induce unfavorable interactions in the folded protein, which reduce the net benefit of the cross-link, and detailed structural information is required for their design.[15] A disulfide bond engineered into cytochrome *c* at positions 20 and 102 in fact does not increase $\Delta G_u°$ compared to the non-cross-linked control ($T_{102}$).[16] In contrast, the β-sheet offers both the geometry and rigidity required for metal ion chelating by dihistidine sites.[1b,17] Furthermore, β-sheets are common elements of secondary structure and can be identified from primary sequence information alone.[18] Thus the ruthenium cross-linking stabilization strategy reported here should be readily applicable to other proteins.

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(9) $\Delta G_u°$ values were determined by GdmCl denaturation, as described previously: Kellis, J. T.; Todd, R. J.; Arnold, F. H. *Bio/Technology* **1991**, *9*, 994–995. Unfolding was measured by monitoring fluorescence (at 346 nm, using an excitation wavelength of 280 nm) or visible absorbance (410 nm). Midpoint melting temperatures ($T_m$) were also determined by measuring the loss of ellipticity at 222 nm of a 20 μM protein solution in 50 mM $NaP_i$, pH 7.0 on a J-600 spectropolarimeter (Jasco, Japan).

(10) In contrast, an exchange-labile metal ion, $Cu^{II}$, stabilizes the $H_{39}H_{58}$ chelating cytochrome *c* by ~2 kcal mol,[1b] and the mechanism appears to be preferential binding of the metal ion to the folded state (ref cited in note 9).

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(13) This view is, of course, oversimplified; there is evidence for enthalpic contributions from cross-linking in both the native and the denatured states of proteins: Kuroki, R.; Inaka, K.; Taniyama, Y.; Kidokoro, S.; Matsushima, M.; Kikuchi, M.; Yutani, K. *Biochemistry* **1992**, *31*, 8323–8328. Doig, A. J.; Williams, D. H. *J. Mol. Biol.* **1991**, *217*, 389–398.

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