Directed Denaturation: Room Temperature and Stoichiometric Unfolding of Cytochrome *c* by a Metalloporphyrin Dimer

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Protein denaturation normally requires elevated temperatures, high concentrations of denaturants such as guanidinium hydrochloride, urea, and various detergents or drastic changes in pH.[1] Other than the chaperones or related proteins,[2] there are very few examples of molecules that unfold target proteins at stoichiometric concentrations under physiologically relevant conditions.[3] In the present paper, we report synthetic agents that selectively denature cytochrome *c* at or near room temperature.

We have previously described molecules that bind to protein exteriors by matching hydrophobic and charged domains over a large surface area of the target protein.[4] In the case of anionic tetraphenylporphyrin derivatives **1a** and **2a** and cytochrome *c*, we observed not only strong binding[4b] to the native state but also a sizable decrease in the melting temperature ($\Delta T_m \approx 20$ °C) of the protein.[5] These results opened the possibility that appropriate modification of the porphyrins might lead to an agent able to fully denature cytochrome *c* at room temperature and under physiological conditions.
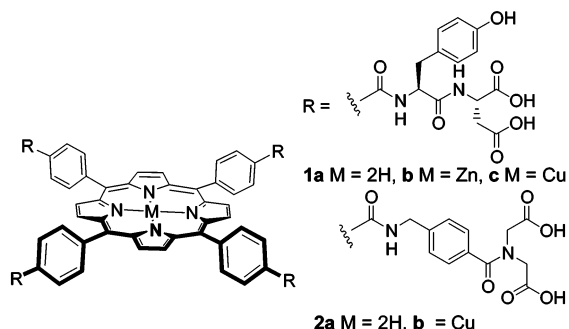
A hint of the chemical features that would be necessary for room-temperature denaturation came in our studies on the effect of pH on the interaction of **1a** with cytochrome *c*. At pH 7.4, **1a** forms a 1:1 complex ($K_d = 20$ nM) with the cationic heme edge region of cytochrome *c* and causes a decrease in the T_m from 85 to 65 °C.[4b,5] In contrast, at room temperature, **1a**, even in excess, had little effect on the native conformation of the protein (as determined by CD). However, at pH 5.8 and room temperature, titration of **1a** into a solution of cytochrome *c* in 5 mM phosphate buffer led to a loss of the CD signal at 222 nm that reached saturation at 4 equiv and corresponded to an unfolding of the tertiary structure of the protein (Figure 1). pH 5.8 buffer had no effect on the thermal stability of cytochrome *c* alone, suggesting the denaturing properties of **1a** at low pH must be due not to a change in the protein but in the chemical behavior of the porphyrin. Indeed, the Soret band of **1a** showed a significant broadening at pH 5.8 relative to 7.4 that could be reversed by addition of acetone.[6] This is consistent with a degree of self-association at the lower pH and suggests that the denaturation effect of **1a** may be due to an aggregated species.[7]
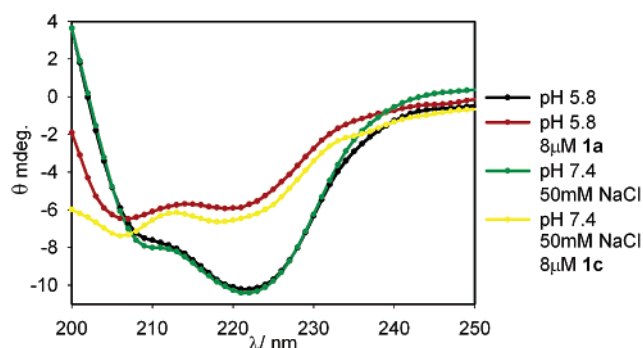

Figure 1. CD spectra of 2 μM cytochrome *c* in the presence and absence of porphyrin receptors **1a** and **1c** at different pH's.

To probe the effects of porphyrin aggregation on the unfolding of cytochrome *c*, we prepared different metalloporphyrin derivatives. Water-soluble zinc(II)-porphyrins have been shown to exhibit minimal aggregation in aqueous buffer[7] due to axial coordination of water to the metal. Consistent with this, **1b** showed no difference in its Soret band at pH's 7.4 and 5.8 or on addition of acetone. At pH 7.4, zinc complex **1b** behaves similarly to **1a** or **2a**, forming a strong 1:1 complex with cytochrome *c* ($K_d = 28$ nM) and lowering the T_m by ∼20 °C. In contrast to **1a**, however, **1b** has no effect on the CD of cytochrome *c* at room temperature even in large excess at pH 5.8. These results suggest that for **1a** at pH 7.4 or zinc derivative **1b** at both pH 5.8 and 7.4, strong binding but weakly denaturing monomeric porphyrins are the dominant porphyrin species interacting with the protein.

To enhance the aggregation of the functionalized porphyrins, we prepared the Cu(II) derivatives **1c** and **2b**. Copper(II) porphyrins are known to undergo extensive dimerization in water[7a] through enhanced $\pi-\pi$ stacking interactions.[8] The UV−vis spectrum of **1c** shows a broadened Soret band at pH 7.4, that sharpens upon addition of acetone, suggesting the presence of aggregated porphyrins. An exciton coupling band in the CD spectrum of **1c** that decreases upon addition of acetone as well as an isosbestic point in dilution studies indicate that a porphyrin dimer is the predominant species in solution. Because the Cu(II)-porphyrins do not fluoresce, UV-difference spectroscopy was used to show that **2b** forms a 2:1 complex (confirmed by Job analysis) with cytochrome *c* with a K_d value of 60 nM (at pH 7.4, 5 mM phosphate).[9] The thermal denaturation curve for cytochrome *c* in the presence of 2 equiv of **2b** (Figure 2) shows a 50 °C decrease in T_m to 35 °C and confirms the enhanced effect of the porphyrin dimer as compared to the monomer. The effect is even more dramatic with **1c** and required an increase in ionic strength of the solution to derive accurate binding data. Cu complex **1c** bound cytochrome *c* with 2:1 stoichiometry (Job plot) and a K_d of 600 nM (5 mM phosphate, 50 mM NaCl, pH 7.4).[9] Two equivalents of **1c** at room temperature (pH 7.4, 50 mM NaCl, 5 mM phosphate) led to a loss of the CD
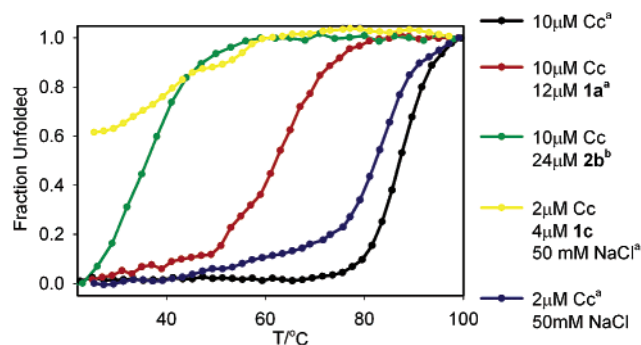
Figure 2. Thermal unfolding profile for cytochrome *c* as a function of added porphyrin receptor (5 mM phosphate, pH 7.4). CD spectra recorded at (a) 222 nm and (b) 410 nm.

signal at 222 nm and formation of a spectrum (Figure 1) that closely resembles that of thermally denatured (>90°) cytochrome *c*. This effect reaches saturation between 1 and 2 equiv of dimer and is not observed for either **1a** or **1b**. In the thermal denaturation experiment, cytochrome *c* in the presence of **1c** (2 equiv) is almost fully unfolded over the whole temperature range (Figure 2).

Furthermore, these large denaturation effects are selective for cytochrome *c* as compared to certain other proteins with a range of pI and T_m values. Figure 3a−e shows the thermal unfolding profiles of α-lactalbumin, Bcl-x$_L$, cytochrome c551, myoglobin, and RNase A in the presence and absence of 2 equiv of **1c**. None of these proteins shows a decrease in T_m of more than 15 °C, while that of cytochrome *c* is >50 °C (Figure 3f) under equivalent conditions. Notably, cytochrome c551, which has a similar structure to cytochrome *c* but different charge distribution, shows almost no change in T_m on addition of **1c**.
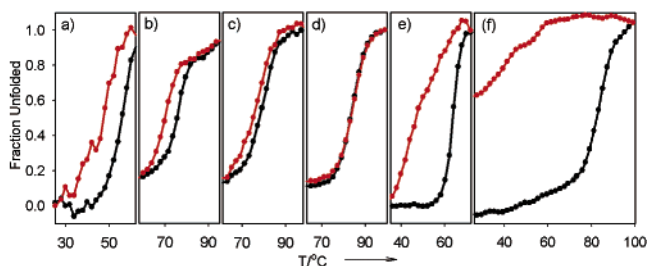

Figure 3. Melting curves for 2 μM protein (5 mM phosphate, 50 mM NaCl, pH 7.4) (in black alone, in red with 4 μM **1c**): (a) α-lactalbumin, (b) Bcl-x$_L$, (c) cytochrome c551, (d) myoglobin, (e) RNase A, (f) cytochrome *c*.

Cytochrome *c* is known to undergo more rapid proteolysis in the denatured state as compared to the compact native form.[10] We therefore assessed the full extent of the unraveling of cytochrome *c* by **1c** at room temperature by measuring the sensitivity of the protein to proteolysis by trypsin. SDS-PAGE analysis of cytochrome *c* cleavage by trypsin alone[11] (Figure 4a) shows very little change in the integrity of the folded protein after 60 min at room temperature. However, in the presence of 4 equiv of **1c**, tryptic digestion of cytochrome *c* is advanced after 15 min and complete after 60 min (Figure 4b). In contrast, 4 equiv of porphyrin **1a**, which under the same conditions is monomeric and thus weakly denaturing, has little effect on the rate of trypsin cleavage of the protein (Figure 4c). These results point to a specific denaturing effect for dimeric **1c** that leads not only to a disruption of the tertiary structure of cytochrome *c* but also to an enhanced rate of proteolysis. Again, selectivity of this effect is suggested both by the unaffected catalytic

activity of trypsin and by the minimal acceleration in cleavage rate of myoglobin and α-lactalbumin (data not shown) on addition of 4 equiv of **1c**.
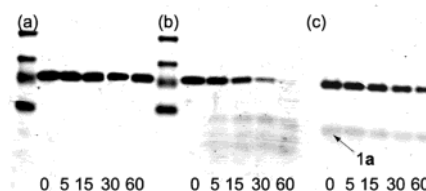

Figure 4. SDS-PAGE analysis of the trypsin proteolysis of 20 μM cytochrome *c* (5 mM phosphate, 50 mM NaCl, pH 7.4) followed over 60 min at room temperature: (a) alone, (b) 80 μM **1c**, (c) 80 μM **1a**.

At this point, the exact mechanism of cytochrome *c* unfolding by **1c** and **2b** has not been determined. However, the large denaturation effect of the Cu porphyrin dimers suggests a preferential interaction with the unfolded state of the protein relative to the folded form. Presumably, the increased size and overall charge of the porphyrin dimers, relative to monomeric **1a**, **1b**, and **2a**, leads to stronger interaction with the larger hydrophobic surface area of the unfolded protein. Selectivity appears to derive from a charge matching of surface residues on the protein with the porphyrin dimer. Some degree of cooperativity between the two porphyrins bound to the denatured state as a dimer must also be considered to explain why 2 equiv of monomeric porphyrin do not bind as well to the denatured state as the dimer. We are currently investigating the interaction of **1c** and **2c** with molten globule and unfolded forms of cytochrome *c* formed by mutated or chemically modified derivatives.

Supporting Information Available: Synthetic procedures and analytical data, UV−vis spectra describing dimerization studies, titration data, and Job plots (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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