Designing Protein Denaturants: Synthetic Agents Induce Cytochrome *c* Unfolding at Low Concentrations and Stoichiometries**

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In contrast to active site inhibitors, very little attention has been given to the design of molecules that alter protein function by changing the native conformation of the target. Such "conformational drugs" could act by returning a misfolded (or mutant) protein to its native state, thereby leading to a gain of function (as has been demonstrated recently with mutated p53[1]), or by inducing a conformational change in an active protein with consequent loss of function.[2] An extreme example of the second case would be a molecule that, on binding to a noncatalytic region of the protein, would force an unraveling of its tertiary and secondary structure.[3] To be effective, such a denaturant should act selectively at near stoichiometric levels and at physiological (micromolar or lower) concentrations.[4, 5] Herein, we describe a class of synthetic ligands that function in this way by binding tightly to and dramatically lowering the melting temperature of cytochrome *c*, a protein commonly used in unfolding studies.

At physiological pH, cytochrome *c* (cyt. *c*), in common with most proteins, requires molar concentrations of guanidinium chloride or urea for denaturation. Lower concentrations of certain phospholipids[6] and surfactants[7] can be effective in destabilizing cyt. *c* but usually at a large excess relative to the protein and at concentrations close to or above their critical micellar concentration (c.m.c.). That stoichiometric unfolding of cyt. *c* might be possible is suggested by the thermodynamic consequences of its binding to cytochrome *c* peroxidase[8] and cytochrome oxidase.[9] Both of these proteins bind tightly to cyt. *c* through complementary protein–protein interactions and cause a 20–27 K decrease in its melting temperature. Thus, a synthetic molecule that matches the distribution of charged and hydrophobic residues on a surface region of cyt. *c* might mimic the function of the natural protein partners and lower the temperature at which it denatures.

The principal protein–protein interaction site on cyt. *c* involves a hydrophobic patch near the heme edge region that is surrounded by several lysine residues (Figure 1a).[10] Compound **1** (Scheme 1), containing four anionic Tyr–Asp substituents around a central porphyrin ring, provides a good match for this distribution of functionality and binds tightly to the heme edge region of cyt. *c* ($K_d = 20$ nM).[11] Moreover, CD studies at room temperature indicated that binding of **1** leads to no detectable change in the secondary structure of the cyt. *c*. However, the thermal stability of the protein is dramatically affected. At pH 7.4, cyt. *c* (at 10 μM) shows a melting temperature (T_m) of 85 °C, as monitored by the loss of the CD signal at 222 nm (Figure 2). Upon addition of 1.2 equivalents of **1**, the T_m dropped to 64 °C. Increasing the concentration of **1** to 60 μM (6 equiv) lead to a further decrease in T_m to 53 °C, corresponding to a 32 K reduction relative to the unbound protein. A single equivalent of **1**, however, is all that is needed to achieve this effect. At 70 °C, titration of **1** into a 10 μM solution of cyt. *c* leads to complete denaturation of the protein at 10 μM (Figure 3).

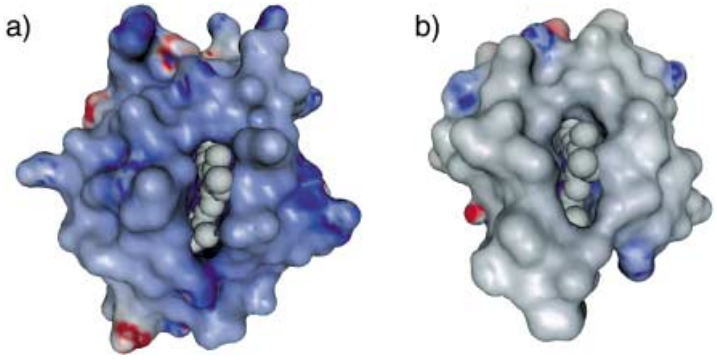
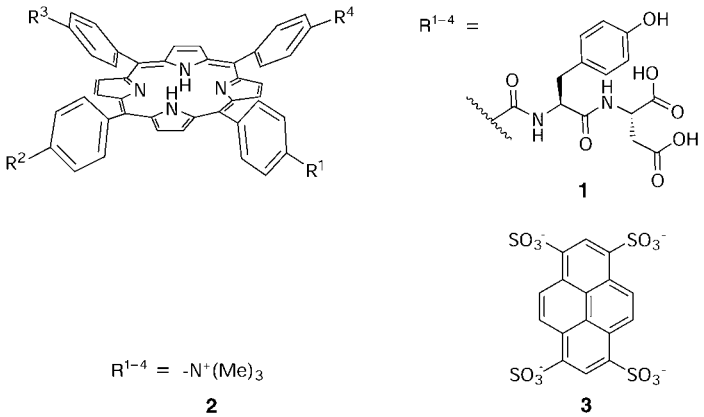


Figure 1. a) X-ray crystal structure of horse cyt. *c*, and b) *Pseudomonas aeruginosa* cyt. *c*551 viewed towards the heme edge region and displayed as surface models color coded by electrostatic (red negative, blue positive) surface potential.



Scheme 1. Chemical formulas of compounds **1**–**3** employed in the denaturation studies.

Extending the thermal denaturation to 100 °C resulted in poor thermodynamic reversibility of cyt. *c* (~40%) due to irreversible aggregation and misligation of heme.[12] However, the lower temperatures required for the thermal transition of cyt. *c* in the presence of **1** lead to higher levels of reversibility (~90%). If the irreversible step, according to the Lumry–Eyring model,[13] proceeds at significant rates only at temperatures above the T_m, then the application of equilibrium thermodynamics will not lead to large errors.[14, 15] Thus, the $\Delta\Delta G$ value, based on the decrease in T_m (21 K) when compound **1** is present at 1.2 equivalents, is approximately 5 kcal mol^{-1}.

The importance of matching protein surface features with recognition sites on the synthetic agent was emphasized by molecules with modified charge or hydrophobic characteristics. For example, tetrakis-*p*-trimethylaminophenylporphine, tetratosylate **2**[16] is charge-mismatched relative to

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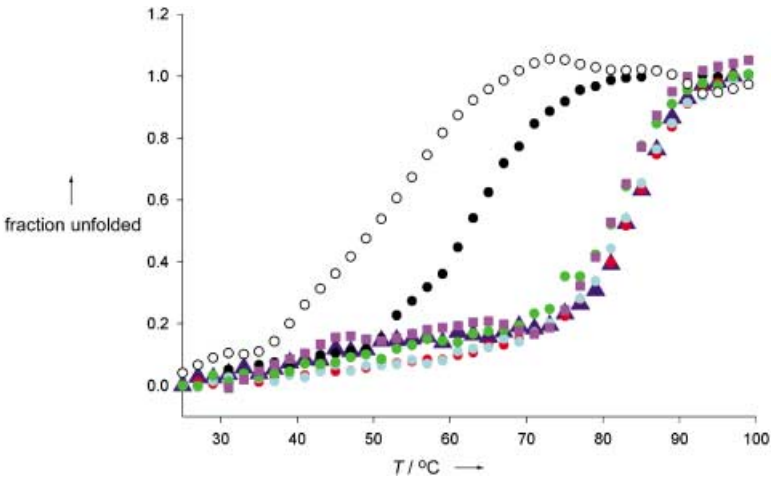

Figure 2. CD-monitored thermal denaturation profiles (θ at 222 nm). ▲ = cyt. *c* (10 μM); ● = cyt. *c* + **1** (10 μM + 12 μM); ○ = cyt. *c* + **1** (10 μM + 60 μM); ● = cyt. *c* + **3** (10 μM + 12 μM); ● = cyt. *c* + **3** (10 μM + 60 μM); ● = cyt. *c* + SDS (10 μM + 12 μM); ■ = cyt. *c* + SDS (10 μM + 120 μM). All experiments were carried out in a sodium phosphate buffer (5 mM, pH 7.4).

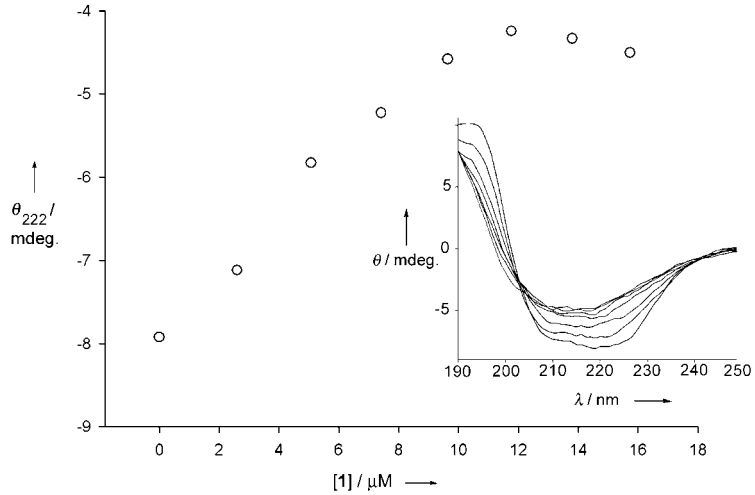

Figure 3. CD-monitored thermal denaturation profiles (θ_{222}) of cyt. *c* (10 μM) as a function of added **1** at 70 °C. Inset: Far-UV CD spectrum (190–250 nm) of cyt. *c* at increasing concentrations of **1**. All experiments were carried out in a sodium phosphate buffer (5 mM, pH 7.4).

cytochrome *c* and shows no effect on its T_m even at 60 μM concentrations (not shown). Tetrasulfonatopyrene tetra-sodium salt **3** serves as an anionic nonpophyrinoid compound, with appreciable hydrophobic surface area and binds moderately to cyt. *c* ($K_d = 8$ μM) but has almost no effect on its thermal denaturation profile (Figure 2). The destabilizing influence of **1** on cyt. *c* is not due to nonspecific surfactant effects. At concentrations between 12 to 120 μM, sodium dodecyl sulfate (SDS) did not affect the T_m of the protein (Figure 2). Previous reports have shown that SDS can denature cyt. *c* but at higher concentrations, close to the c.m.c. value of 2.3 mM.[7] The UV absorption of **1** follows Beer's law, and no large aggregate formation was detected by resonance light scattering, indicating that the denaturing effect is due to discrete porphyrin monomers.

The importance of electrostatics in the denaturing effects of **1** was probed by studying proteins structurally related to native horse-heart cytochrome *c*. A modified cyt. *c*, where 60% of the lysine ε-amino groups are acetylated, has a very different surface charge distribution from the native protein but maintains a similar level of secondary structure at pH 7.4 and 25 °C albeit with a lower melting temperature (65 °C).[17] Compound **1** binds much more weakly to acetylated cytochrome *c* ($K_d > 50$ μM from fluorescence titration) and causes no change in its denaturation profile (Figure 4). These results point to an important role for the lysine residues in cyt. *c* for both the strong binding and destabilizing effects of **1**. Finally, we studied the effect of **1** on cytochrome *c*551 from *P. aeruginosa*[18] (also known as cyt. *c*8) that has a similar structure to horse cyt. *c* but lacks the extensive cationic patch around the heme crevice (Figure 1b). The affinity of **1** for cyt. *c*551 ($K_d = 20$ μM) was 10^3 fold weaker than horse cyt. *c*, again pointing to a key role for the clustered lysine residues. However, the T_m from the thermal denaturation of cyt. *c*551 (82 °C) closely resembles that of horse cyt. *c* and showed no change (Figure 4) on addition of **1** (12 μM). The lack of any effect on a closely related protein points to a degree of specificity in the action of **1** as a denaturant imposed by the matching of recognition surfaces.

The exact origin of this denaturing effect remains to be determined; however, tight binding of the denaturant to the native conformation of cyt. *c* seems to be a requirement. But how does that lead to unfolding of the protein? Charged amino acid residues on the surface of cyt. *c* (and many other proteins) are known to stabilize the native conformation.[19] Charge optimization is believed to be a general mechanism for stability enhancement amongst thermophiles[20, 21] and more recently has been applied to engineering highly thermostable proteins from mesophilic proteins.[22, 23]

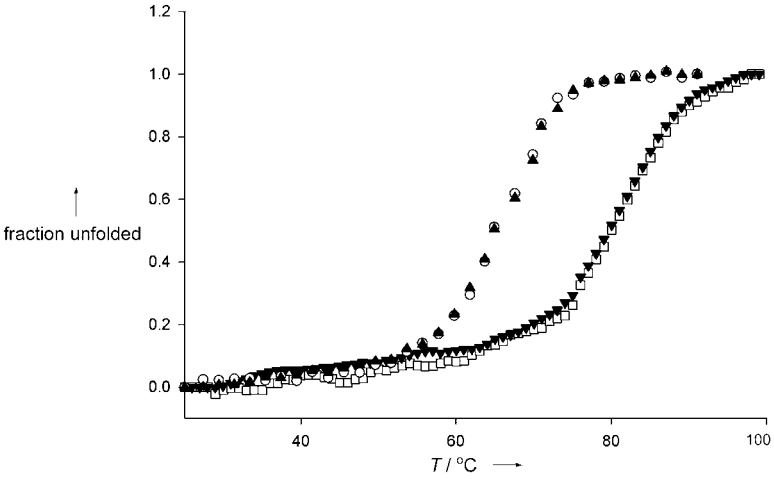

Figure 4. CD-monitored thermal denaturation profiles (θ_{222}). □ = *P. aeruginosa* cyt. *c* (10 μM); ▼ = *P. aeruginosa* cyt. *c* + **1** (10 μM + 12 μM); ○ = acetylated horse cyt. *c* (10 μm); ▲ = acetylated horse cytochrome *c* + **1** (10 μM + 12 μM). All experiments were carried out at pH 7.4, 5 mM sodium phosphate buffer.

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Horse cyt. *c* is a highly basic protein with a pI close to 10, where the charge state of the protein at pH 7 is +8. Association of **1**, an octaanionic species near neutral pH, leads to a major disruption of the coulombic interactions between the surface lysine residues and other regions of the protein with likely destabilization of the folded structure. In a related way, partial acetylation of the lysine (see above)[17] and binding to acidic lipid membranes[24] leads to a disruption of electrostatic surface potential and a similar destabilization of cyt. *c*. However, interaction with the denatured state may make an equally significant contribution to the effect of **1**.[25, 26] In order to probe the interaction of **1** with the denatured state, we carried out a fluorescence quenching titration at higher temperatures. At 75 °C, the fluorescence of **1** is completely quenched by addition of approximately 0.33 equivalents of cyt. *c*. This indicates binding of at least 3 equivalents of **1** to the unfolded form of the protein, which would be expected to have a larger accessible hydrophobic surface area than the native conformation.[27]

Compound **1** takes advantage of complementary hydrophobic and electrostatic interactions to achieve high affinity and selectivity. Like other destabilizers,[5] **1** appears to act through destabilizion of the native state in addition to the stabilization of the unfolded state. However, in the case of **1**, increased selectivity and efficacy is achieved through strong affinity to both states, inducing unfolding at much lower concentrations. This strategy offers a potentially general approach to designing molecules that denature other globular proteins with potency and selectivity.

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Synthesis and Characterization of Ag_2NiO_2 Showing an Uncommon Charge Distribution**

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The unique structural features of silver-rich silver(I) oxides were the earliest experimental evidence suggesting attractive $d^{10}-d^{10}$ interactions between monovalent silver atoms.[1, 2] In spite of bearing equal positive charges, the silver atoms in these compounds interconnect forming cluster-like ensembles corresponding to sections of the element structure of silver. Those structural features are accompanied by the formation of an empty band of mainly Ag-5s character near the Fermi level, capable of accommodating additional electrons. Consequently, silver is expected to adopt subvalent valence states in solid materials.[3] Only a few examples such as, Ag_2F,[4] Ag_3O,[5] Ag_5SiO_4,[6, 7] Ag_5GeO_4,[6, 8] and $Ag_5Pb_2O_6$[9] have been prepared to date. The properties range from metallic behavior in Ag_2F, Ag_3O, and $Ag_5Pb_2O_6$ to semiconducting in Ag_5SiO_4 and Ag_5GeO_4.

Surprisingly, the new silveroxonickelate Ag_2NiO_2 also contains subvalent silver. Conventionally, the oxidation states +1 and +2 would be assigned to silver and nickel, respectively, which correspond to the valence distribution in the homologous compound Ag_2PdO_2.[10] These assignments, however, severely conflict with the structural features, magnetic moment, and spectroscopic properties found in Ag_2NiO_2. Instead, all the experimental findings are consistent with a charge distribution of $[Ag_2]^+[NiO_2]^-$.

Ag_2NiO_2 has been prepared by the solid-state reaction of Ag_2O and NiO under high oxygen pressure. Ag_2NiO_2 is a lustrous black solid and insensitive to air and water. The crystal structure has been determined and refined from X-ray and neutron powder data, and from single-crystal X-ray data.[11] There are striking similarities with the delafossite structure[12] on the one hand as well as with the silver suboxide Ag_3O[5] and the silver subfluoride Ag_2F,[4] on the other.

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