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Disruption of protein–protein interactions: design of a synthetic receptor that blocks the binding of cytochrome *c* to cytochrome *c* peroxidase

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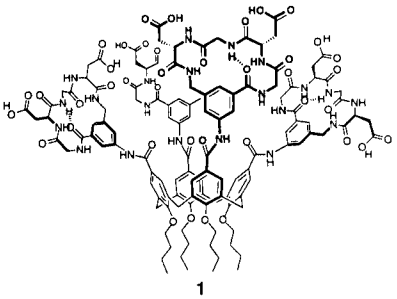
Synthetic receptor 1 has been found *via* fluorescence titration to compete effectively with cytochrome *c* peroxidase for binding cytochrome *c* (Cc), forming 1:1 Cc:1 complex with a binding constant of (3 ± 1) × 10^8 M^{-1}, and to disrupt Cc:Apaf-1 complex, a key adduct in apoptosis.

Protein–protein recognition is critical to virtually all cellular processes. Consequently, molecules which could specifically disrupt the high affinity natural protein complexes found physiologically could provide an important route to modulate cellular processes. However, there are few examples of such a modulation.[1] Since high affinity protein–protein complexes generally involve large surface areas,[2] it has been argued that small synthetic molecules which bind at the interaction surface are unlikely to compete with high affinity natural protein partners. Here, we show that a synthetic receptor molecule of structure **1** can bind cytochrome *c* (Cc) with a sufficiently high



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affinity to displace a natural high affinity protein partner, cytochrome *c* peroxidase (CcP). Cytochrome *c* provides an important model for such studies for several reasons. First, the structure of Cc is well characterized.[3] Second, the Cc:CcP complex is unusually well characterized, both from structural and thermodynamic viewpoints.[4,5] Finally, the key role of Cc in mediating apoptosis[6–8] makes it a particularly interesting target.

Several previous studies have explored the binding of small molecules to Cc.[3,9] However, in few previous cases was the binding sufficiently strong and specific to be shown to compete with the binding of natural partner proteins at relevant concentrations ($< 10^{-6}$ M). Preliminary data on Cc binding by **1** suggested the possibility of specific and high affinity binding.[10] To test this possibility, we have carried out a series of spectroscopic studies and established that the synthetic receptor **1** is indeed capable of disrupting the physiological complex of Cc with CcP by binding competitively to Cc. From the concentration of **1** needed to disrupt the Cc:CcP binding, we can estimate that the dissociation constant (K_d) for the Cc:**1** complex is near the nanomolar level ($\sim 10^{-8}$ M) in a phosphate buffer (10 mM, pH 6.0). Furthermore, the competitive displacement of CcP from its natural protein partner by **1** suggests that they share a similar binding site on the surface of Cc.

The competitive binding experiments were based on the quenching of strong fluorescence of magnesium(II) CcP (MgCcP) by Cc and the reversal of that quenching by addition of receptor **1**. The reaction schemes are illustrated in eqns. (1) and (2). It has been demonstrated that the magnesium substitution of iron in the native CcP does not alter the binding characteristics of CcP with Cc.[5] The use of MgCcP is advantageous because of its strong, interference-free fluorescence signals. In a typical experimental procedure, 3.0 mL of 0.077 μM solution of MgCcP[5] in 10 mM degassed potassium phosphate buffer (KPhos, pH 6.0) was prepared in a quartz cuvette of 1 cm pathlength equipped with magnetic stirrer. The fluorescence spectrum of the solution was recorded on a PTI fluorimeter (Photon Technology International, NJ) with an excitation wavelength of 556 nm and a scan rate of 1 nm s^{-1} at 15.0 °C (Fig. 1a). The solution was titrated *in situ* with a 3.5 μM solution of yeast Cc (Sigma) to an equivalent point [CcP]:[Cc] = 1. Upon Cc:CcP complex formation, the fluorescence signals of MgCcP at 598 and 653 nm were quenched by resonant energy transfer (Fig. 1b). Then a 4.48 μM solution of receptor **1**[10] in 10 mM KPhos (pH 6.0) was added in aliquots of 10 to 20 μL. The fluorescence spectrum was measured after each addition until the [**1**]:[Cc] molar ratio reached 5 or higher (Figs. 1c and 1d). Upon addition of receptor **1**, the intensity of the fluorescence signals of MgCcP increased, indicating that CcP is gradually freed from the Cc:CcP complex.

$$\mathrm{Cc} + \mathrm{CcP} \leftrightharpoons \mathrm{Cc{:}CcP} \quad (1)$$

$$\mathrm{Cc{:}CcP} + \mathbf{1} \leftrightharpoons \mathrm{Cc{:}}\mathbf{1} + \mathrm{CcP} \quad (2)$$

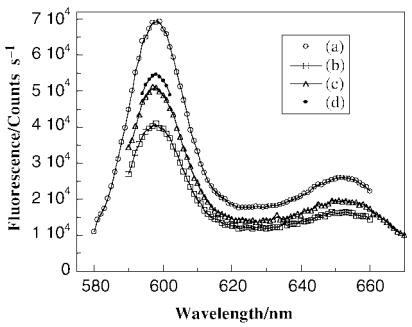

Fig. 1 Representative fluorescence spectra of (a) MgCcP (0.075 μM), (b) MgCcP (0.075 μM) with 0.077 μM of Cc, and the MgCcP:Cc complex titrated with (c) 0.11 and (d) 0.28 μM of **1**. Excitation at 556 nm at 15.0 °C in 10 mM KPhos buffer (pH 6.0).

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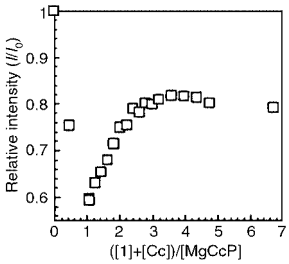

Fig. 2 Plots of the relative fluorescence intensity (I/I_o) at 598 nm against the normalized concentration ([Cc] + [**1**])/[CcP] (ex. 556 nm). [Cc] = 0.077 μM, [MgCcP] = 0.075 μM in 10 mM KPhos buffer (pH 6.0) at 15.0 °C.

Fig. 2 shows the plots of relative intensity of the fluorescence at 598 nm against the normalized concentration, ([Cc] + [**1**])/[CcP]. At [Cc]/[CcP] = 1 and before addition of **1**, the fluorescence intensity (I) of MgCcP was reduced to ~57% of the initial value (I_o), which is in agreement with that reported in the literature.[5] Addition of **1** resulted in a sharp increase in the relative intensity (I/I_o) until ([Cc] + [**1**])/[CcP] reached about 2, *i.e.* [**1**]/[Cc] ≈ 1, after which the intensity levelled off, suggesting the formation of 1:1 Cc:**1** complex. The intensity does not increase to the original level because of partial photodegradation of MgCcP. The recovery of fluorescence suggests that receptor **1** effectively disrupts the Cc:CcP complex at nanomolar concentrations (*i.e.* ~0.08 μM), and releases CcP free into solution. Under these conditions, the interaction between **1** and Cc appears stronger than that between CcP and Cc, with a K_d value < 100 nM.

The binding constant (K_b) for Cc:**1** complex can be estimated from the competitive titration results using a standard nonlinear curve-fitting. The competition equilibrium constant K_c for eqn. (2) can be expressed [eqn. (4)] in terms of the K_b's for eqn. (1) ($K_{b1} = 2 \times 10^7$ M^{-1})[5] and for eqn. (3) (K_{b2}). Assuming that the concentration of free Cc in eqn. (3) at equilibrium is negligible, a quadratic equation can be derived as shown in eqn. (5), where $A = [Cc]_{Total}$, $B = [CcP]_{Total}$, $x = [\mathbf{1}]_{Total}$, and $y = (I_{obs} - I_f)/(I_i - I_f)$ with I_i and I_f being the fluorescence intensities at ([Cc] + [**1**])/[CcP] of 1 and of ⩾2, respectively. From the solution for y in eqn. (5), K_c was obtained. A set of typical results are shown in Fig. 3, from which K_{b2} was estimated to be $(3 \pm 1) \times 10^8$ M^{-1}.

$$Cc + \mathbf{1} \leftrightharpoons Cc{:}\mathbf{1} \quad (3)$$

$$K_c = K_{b2}/K_{b1} \quad (4)$$

$$(B^2K_c - B^2)y^2 + (K_cBx - ABK_c + AB + B^2)y - AB = 0 \quad (5)$$

The titration of the MgCcP solution with **1** in the absence of Cc showed little change in the fluorescence of MgCcP in the range of [**1**]/[MgCcP] from 0 to 5, indicating that there is no significant interaction between **1** and CcP. The region of Cc

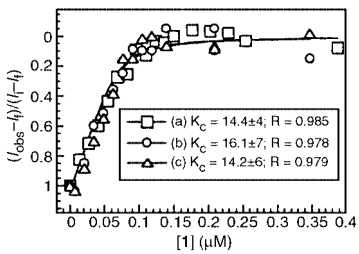

Fig. 3 Typical curve-fitting results for the fluorescence titration of 1:1 Cc:CcP complex with the receptor **1**. The values of $y = (I_{obs} - I_f)/(I_i - I_f)$ for emissions at (a) 598 nm at 15.0 °C, (b) 598 nm at 25.0 °C and (c) 653 nm at 25.0 °C are plotted against the concentration of **1**. The experimental data are fitted according eqn. (5) to estimate K_c values. Excitation at 556 nm, [MgCcP] = 0.076 μM, [Cc] = 0.077 μM in 10 mM KPhos buffer (pH 6.0).

involved in binding to CcP contains an array of positively charged residues that interacts with a complementary patch of negative residues on CcP.[4] The polyanionic receptor **1** competes successfully with CcP, presumably by binding to a similar site on the surface of Cc. The CcP molecules are freed from the interactions with Cc, leading to the observed recovery of fluorescence.

The binding of **1** with Cc has been independently confirmed by direct fluorescence titration of **1**. Receptor **1** exhibits a broad fluorescence peak at ~432–443 nm with excitation wavelength of 298 nm. Addition of Cc results in a rapid decrease in the fluorescence intensity, which levels off as Cc concentration increases. Further quantitative interpretations of such data are precluded at the present time, since the receptor molecules (**1**), due to their surfactant-like structure, tend to aggregate, which interferes with the determination of binding stoichiometry. Reducing the concentration of **1** prevents the aggregation but the decrease in fluorescence signal to noise ratio impedes quantitative analysis.

The ability to bind to Cc with high affinity is of great interest, given the major role of Cc as a signal protein for activating Apaf-1 (the apoptosis protease activating factor-1) protein in apoptosis.[6–8] The binding constant for the 2:1 Cc:Apaf-1 complex was estimated to be ~10^{11} M^{-1} from the fluorescence polarization titration measurements, in which Apaf-1 was added to nanomolar concentrations of horse heart zinc(II) Cc and the increase in polarization was monitored.[8] Upon formation of the Cc:Apaf-1 complex, receptor **1** was added to the system. At a [**1**]/[Cc:Apaf-1] molar ratio of ~200, the relative fluorescence polarization decreased significantly from 1.9 to 1.3. The preliminary data indeed suggest that **1** can disrupt the Cc:Apaf-1 interactions. More detailed results of further studies will be reported in due course.

In conclusion, we have demonstrated that the physiological complex of Cc with CcP can be disrupted by a synthetic receptor (**1**). Receptor **1** competes effectively with CcP for binding Cc, forming 1:1 Cc:**1** complex with a binding constant of ~10^8 M^{-1}. Investigation is in progress to evaluate the effects of the structure of synthetic receptors on their competitive binding with various proteins, including Apaf-1, and protein–protein complexes.

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