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Molecular recognition of a model globular protein apomyoglobin by synthetic receptor cyclodextrin: effect of fluorescence modification of the protein and cavity size of the receptor in the interaction

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Labelling of proteins with some extrinsic probe is unavoidable in molecular biology research. Particularly, spectroscopic studies in the optical region require fluorescence modification of native proteins by attaching polycyclic aromatic fluoroprobe with the proteins under investigation. Our present study aims to address the consequence of the attachment of a fluoroprobe at the protein surface in the molecular recognition of the protein by selectively small model receptor. A spectroscopic study involving apomyoglobin (Apo-Mb) and cyclodextrin (CyD) of various cavity sizes as model globular protein and synthetic receptors, respectively, using steady-state and picosecond-resolved techniques, is detailed here. A study involving Förster resonance energy transfer, between intrinsic amino acid tryptophan (donor) and *N, N*-dimethyl naphthalene moiety of the extrinsic dansyl probes at the surface of Apo-Mb, precisely monitor changes in donor acceptor distance as a consequence of interaction of the protein with CyD having different cavity sizes (β and γ variety). Molecular modelling studies on the interaction of tryptophan and dansyl probe with β-CyD is reported here and found to be consistent with the experimental observations. In order to investigate structural aspects of the interacting protein, we have used circular dichroism spectroscopy. Temperature-dependent circular dichroism studies explore the change in the secondary structure of Apo-Mb in association with CyD, before and after fluorescence modification of the protein. Overall, the study well exemplifies approaches to protein recognition by CyD as a synthetic receptor and offers a cautionary note on the use of hydrophobic fluorescent labels for proteins in biochemical studies involving recognition of molecules. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: molecular recognition; fluorescence labeling; cyclodextrin; apomyoglobin; receptor cavity size

INTRODUCTION

Molecular recognition is a fundamental step crucial in any biological processes starting from cellular signaling to enzyme catalysis, and essentially involves the recognition between two or more molecular binding partners, leading either to their association or to their rejection (Baron and McCammon, 2013). How and why two molecular binding partners specifically fit together among a plethora of molecular components in the crowded cell environment, and the weak intermolecular forces that are on them, are questions of the complementarity of size, shape, and chemical surface (Hof and Rebek, 2002). Proteins are important target molecules for recognition by various signaling molecules. Visualization of the *in vitro* complex cellular processes involving proteins requires the use of spectroscopically distinguishable fluorescent reporters. Labelling of proteins by using various hydrophobic fluorescent probes is a procedure often followed in biochemical studies involving the recognition of molecules. The hydrophobic fluorescent probe dansylation chloride is widely used to modify charged polar side chains of amino acids similar to lysine and arginine (Banik *et al*., 1993; Mondol *et al*., 2012). However, changes in even one amino acid property of a protein because DNA point mutation may affect the molecular recognition of the protein, leading to various diseases (Warren *et al*., 1998; Ogura *et al*., 2001; Corradini *et al*., 2004; Valente *et al*., 2004; Borroto-Escuela *et al*., 2010). For example, the creation of hydrophobic spot in hemoglobin because of Glu6Val point mutation causes clumping together (polymerization) of hemoglobin molecules into rigid fibers to cause 'sickling' of red blood cells (Voet and Voet, 1995). Nevertheless, labelling of proteins by using hydrophobic fluorescent probe is a common procedure in biochemical studies, whereas its effect on molecular recognition process is less attended in the literature. In the present study, the lysine side chains of apomyoglobin (Apo-Mb) has been dansylated to study the effect of hydrophobic surface modification of the protein on the molecular recognition process by cyclodextrins (CyD) as a model synthetic receptor.

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Cyclodextrins are cyclic oligosaccharides known to recognize a wide variety of organic, as well as inorganic, guest molecules, forming host-guest inclusion complexes with various guest molecules of suitable size fitting the host CyD cavity (Dodziuk, 2006; El-Kemary and Douhal, 2006; Hashidzume and Harada, 2011; Martín *et al*., 2012; Zheng *et al*., 2012). Accordingly, CyDs are used as chiral selectors in chromatographic separations (Li and Purdy, 1992; Cserháti and Forgács, 2003). Inclusion complexation of β-CyD can effectively discriminate the L-phenylalanine and D-phenylalanine, depending on the stabilization energies and inclusion geometries of the complexes (Aree *et al*., 2012). In some significant recent publications from the group of Harada *et al*., the macroscopic molecular recognition property of CyDs have been well demonstrated (Zheng *et al*., 2011; Yamaguchi *et al*., 2012; Zheng *et al*., 2012). α-CyD and β-CyD have even been found to discriminate the subtle differences in the structures of human serum albumin and bovine serum albumin (Oi *et al*., 2011). Small changes in the degree of substitution for a given CyD is known to influence the specific molecular recognition and as such the stability on a specific protein (Samra *et al*., 2010). The study by Douhal et al., shows higher association of the drug milrinone with dimethyl-β-CyD, in comparison with β-CyD (El-Kemary *et al*., 2006). The slow solvation dynamics of water molecules in the CyD cavities, captured in picosecond time domain are clear from the work of Fleming (Vajda *et al*., 1995) and Bhattacharyya (2008). Significant effects are exerted by the cavities on reactions (like proton transfer and charge transfer) that may occur in cellular confinement (Martín *et al*., 2012). Accordingly, CyDs are considered as simple biomimetic compounds, and its interaction with proteins are suitable model systems for biological molecular recognition.

In the present study, we examine the molecular recognition of unmodified and dansyl-modified Apo-Mb by two CyD (β-CyD and γ-CyD) molecules having different hydrophobic cavity size. Steady-state fluorescence spectroscopy and picosecond-resolved Förster resonance energy transfer (FRET) study reveals the molecular recognition of the protein in a precise way. The thermodynamic free energy change (ΔG) and stoichiometry of the association is estimated using the emission data. The structural alteration of the protein in presence of CyD has been studied using circular dichroism (CD) spectroscopy. CD spectroscopy has also elucidated the effect of CyD binding on the thermal denaturation of the proteins. The molecular modelling studies support the experimentally derived observations quite reasonably.

MATERIALS AND METHODS

Apomyoglobin from horse skeletal muscle was purchased from Sigma. β-CyD (Aldrich), γ-CyD (Aldrich), sodium dihydrogen phosphate (Sigma 99%), di-sodium hydrogen phosphate (Sigma), and dansyl chloride (Molecular Probes Inc.) were used as received. Aqueous stock solutions of Apo-Mb were prepared in a phosphate buffer (10 mM) at pH 7.5 by using double distilled water. The working concentration of the protein was 1.66 μM, far below the aggregation concentration of the protein (Tcherkasskaya and Ptitsyn, 1999; Stevens *et al*., 2010).

Labeling of Apo-Mb with dansyl chloride was carried out by incubating 1.66 μM of the protein in 10 mM phosphate buffer at pH 7.5, with a 10-fold molar excess of dansyl chloride (in acetonitrile) at 4°C, for overnight stirring. The reaction was quenched by adding Tris–HCl, pH 7.5. It was then dialyzed against 10 mM phosphate buffer, pH 7.5 (Banik *et al*., 1993). The reactive groups of amino acids (lysine and arginine) react with dansyl chloride in their deprotonated form as a nucleophile. Because the pK_a of arginine ($pK_a = 12.48$) is greater than that of lysine ($pK_a = 10.5$), at pH 7.5 lysine is favorably dansylated (Haugland, 1996; Mondol *et al*., 2012).

Far ultraviolet CD measurements were performed on a JASCO 815 spectrometer (from JASCO International Co. Ltd, Tokyo, Japan) by using a 0.2 cm path length cell. Emission spectra were recorded with a Horiba Jobin Yvon Fluorolog-3 fluorimeter (from Horiba Ltd, Kyoto, Japan). Fluorescence transients were measured using commercially available spectrophotometer (LifeSpec-ps) from Edinburgh Instrument, UK. For 293 nm laser excitation, we used a femtosecond-coupled time-correlated single-photon counting setup described earlier (Rakshit *et al*., 2012; Saha *et al*., 2013). Briefly, the samples were excited by the third harmonic laser beam (293 nm) of the 880 nm (0.5 nJ per pulse) by using a mode-locked Ti-sapphire laser with an 80 MHz repetition rate (Tsunami, Spectra Physics), pumped by a 10 W Millennia (Spectra Physics) followed by a pulse-peaker (rate 8 MHz), and a third harmonic generator (Spectra-Physics, model 3980). The third harmonic beam was used for excitation of the sample inside the time-correlated single-photon counting instrument (instrument response function (IRF) = 70 ps) and the second harmonic beam was collected for the start pulse. Fluorescence transients were fitted by a nonlinear least square fitting procedure to a function $\left(X(t) = \int_0^t E(t')R(t-t')dt'\right)$ comprising the convolution of the IRF (E(t)) with a sum of exponentials $\left(R(t) = A + \sum_{i=1}^{N} B_i e^{-t/\tau_i}\right)$ with pre-exponential factors (B_i), characteristic lifetimes (τ_i), and a background (A). Relative concentration in a multi-exponential decay was finally expressed as $c_n = \left(B_n / \sum_{i=1}^{N} B_i\right) \times 100$. The quality of the curve fitting is evaluated by a reduced chi-square and residual data.

In order to estimate FRET efficiency of the donor (trp) and hence to determine the distance of donor-acceptor pairs, we used the following methodology (Lakowicz, 2006). The Förster distance (R_0) in Å is given by

$$R_0 = 0.211 \times \left[\kappa^2 n^{-4} Q_D J(\lambda)\right]^{\frac{1}{6}} \quad (1)$$

where κ^2 is a factor describing the relative orientation in space of the transition dipoles of the donor and acceptor. For donor and acceptors that are randomized by rotational diffusion prior to energy transfer, the magnitude of κ^2 is assumed to be 2/3. The refractive index (n) of the medium is assumed to be 1.4 (Lakowicz, 2006). Q_D, the quantum yield of the donor in the absence of acceptor, was taken to be 0.18 (Stevens *et al*., 2010); J(λ), the overlap integral, which expresses the degree of spectral overlap between the donor emission and the acceptor absorption, is given by

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_D(\lambda)d\lambda} \quad (2)$$

where $F_D(\lambda)$ is the fluorescence intensity of the donor in the wavelength range of λ to λ + dλ and is dimensionless. $\varepsilon_A(\lambda)$ is the extinction coefficient (in $M^{-1}\,cm^{-1}$) of the acceptor at λ. If λ is in nm, then J(λ) is in units of $M^{-1}\,cm^{-1}\,nm^4$. Once the value of R_0 is known, the donor-acceptor distance (r) can be easily calculated using the formula

$$r^6 = \frac{R_0^6(1-E)}{E} \quad (3)$$

where E is the efficiency of energy transfer. The transfer efficiency (E) is

measured using the fluorescence lifetime of the donor, in absence (τ_D) and presence (τ_{DA}) of the acceptor.

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad (4)$$

Distance distribution between donor and acceptor was estimated according to the procedure described in the literature (Lakowicz, 2006; Batabyal *et al.*, 2013). The observed fluorescence transients of the donor molecules in the absence of an acceptor were fitted using a nonlinear least-squares fitting procedure (software SCIENTIST) to the following function

$$I_D(t) = \int_0^t E(t')p(t'-t)dt' \quad (5)$$

which comprises the convolution of the IRF ((E(t)) with exponential $\left(p(t) = \sum_i \alpha_{Di} \exp(-t/\tau_{Di})\right)$. The convolution of the distance distribution function P(r) in the fluorescence transients of donor in presence of acceptor in the system under studies is estimated using the same software (SCIENTIST) in the following way.

The intensity decay of D-A pair, spaced at a distance r, is given by

$$I_{DA}(r,t) = \sum_i \alpha_{Di} \exp\left[-\frac{t}{\tau_{Di}} - \frac{t}{\tau_{Di}}\left(\frac{R_0}{r}\right)^6\right] \quad (6)$$

and the intensity decay of the sample considering P(r) is given by

$$I_{DA}(t) = \int_{r=0}^{\infty} P(r) I_{DA}(r,t) dr \quad (7)$$

Where P(r) consist of the following terms:

$$P(r) = \frac{1}{\sigma\sqrt{2\pi}} \exp\left[-\frac{1}{2}\left(\frac{\bar{r}-r}{\sigma}\right)^2\right] \quad (8)$$

In this equation, $\bar{r}$ is the mean of the Gaussian with a standard deviation of σ. Usually, distance distributions are described by the full width at half maxima (hw). This half width is given by hw = 2.354σ.

For the molecular modeling studies, we used commercially available ChemBio3D ultra (from CambridgeSoft™) software following the procedure reported earlier (Larsen *et al.*, 1996; Banerjee *et al.*, 2012). The force field employed for these calculations is MM2 with default parameters provided with the software. Conjugate gradient methods were used to search for geometry-optimized structures with a convergence criterion of 0.0001 kcal $Å^{-1}$. The minimized energy structures of the various complexes as investigated in the present study were obtained by the geometry optimization of each component of the complex in vacuum. These geometry-optimized components were then brought together in a face to face orientation and to within Van der Waals radii and re-optimized. Relative binding energies were determined by subtracting the sum of the geometry-optimized energies of the isolated components from the total energy obtained for the geometry-optimized complex.

RESULTS AND DISCUSSIONS

In aqueous solution, tryptophan residues of Apo-Mb has emission maximum at 342 nm (Figure 1a). Gradual addition of β-CyD is associated with a blue shift (24 nm) of the band maxima along with the enhancement of the fluorescence intensity. The blue shift of the emission band is indicative of the reduced local polarity around tryptophan. Consistently, the significant increase in the fluorescence quantum yield indicates the inclusion of tryptophan in more protected hydrophobic interior of the host β-CyD cavity (Scheme 1a) (Khajehpour *et al.*, 2004). The increase in the fluorescence quantum yield is because of the deactivation of the non-radiative decay channels in the hydrophobic cavity of

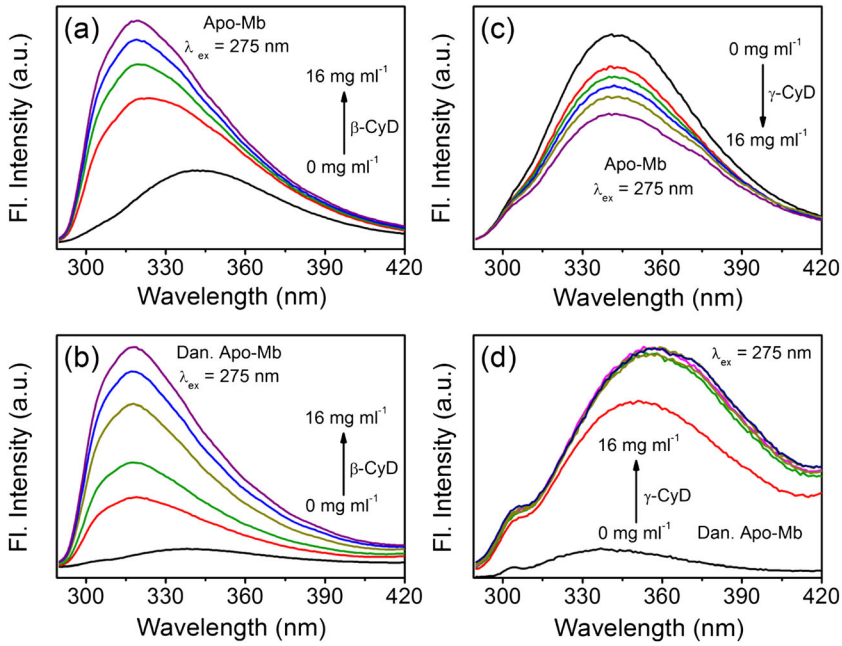
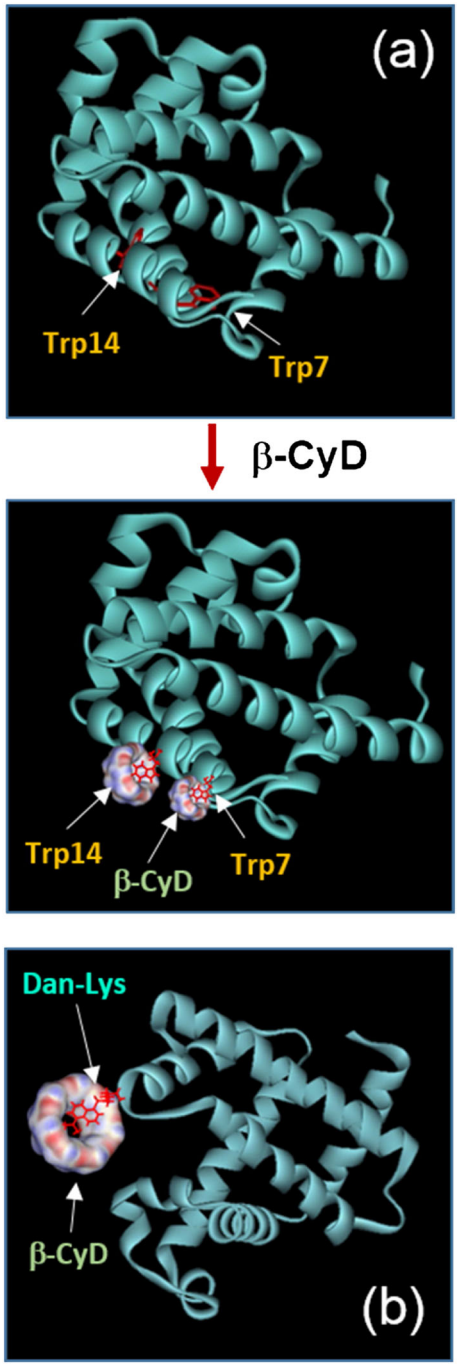


Figure 1. Steady-state emission spectra of tryptophan (λ_{ex} = 275 nm) in dansyl-free (a) and dansyl-modified (b) apomyoglobin with increasing concentrations of β-cyclodextrin. Steady-state emission spectra of tryptophan (λ_{ex} = 275 nm) in dansyl-free (c) and dansyl-modified (d) apomyoglobin with increasing concentrations of γ-cyclodextrin.



Scheme 1. (a) Schematic representation of the molecular recognition of apomyoglobin protein by β-cyclodextrin, through tryptophan inclusion in its hydrophobic cavity. The buried tryptophan side chain is surface exposed to form inclusion complex with β-cyclodextrin. (b) The molecular recognition of apomyoglobin through dansyl inclusion in the hydrophobic cavity of β-cyclodextrin is schematically shown.

β-CyD (Ghosh *et al*., 2011). The observation is well supported by the molecular modelling studies using free tryptophan, showing effective inclusion of the tryptophan side chain in the β-CyD cavity (Figure 2a), with estimated stabilization energy value of $-108\ kJ\ mol^{-1}$. Similar changes in the tryptophan emission is observed even in the dansylated protein (Figure 1b). However, stronger inclusion complexes with β-CyD are formed upon dansylation of the protein (discussed later).

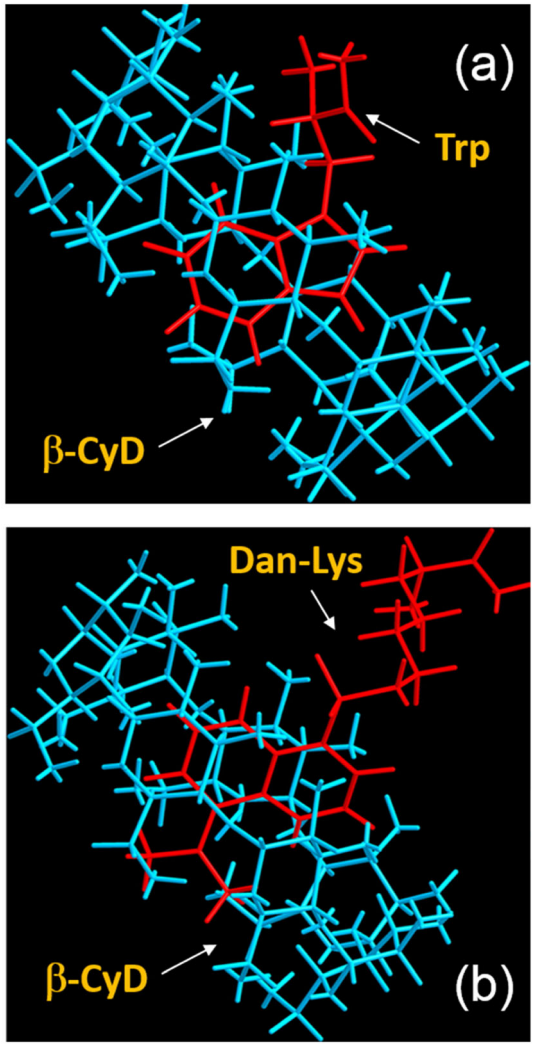


Figure 2. (a) The molecular inclusion complex of tryptophan with β-cyclodextrin. (b) The molecular inclusion complex of dansyl-lysine (Dan-Lys) with β-cyclodextrin. Complexes are minimum energy structures optimized using ChemBio3D™ software.

Molecular recognition in biological systems is specific similar to 'lock and key'. The exact fit of the tryptophan side chains, in the hydrophobic interior of CyD would largely depend upon the size of the molecular cavity. The gradual addition of γ-CyD lowers the emission intensity of tryptophan, without any change in the emission band position (Figure 1c). The unchanged band position of tryptophan indicates that the side chain of tryptophan is not recognized by γ-CyD cavity. Indeed, studies reported so far suggest the insertion of aromatic side chains of peptides into the cavity of natural β-CyD (Otzen *et al*., 2002; Aachmann *et al*., 2003; Danielsson *et al*., 2004) and its derivatives (Matsubara *et al*., 1997; Tang *et al*., 2006). The larger cavity diameter of γ-CyD hardly allows the steric fit of aromatic amino acids in an optimal condition, forming deeper and looser inclusion complex between hydrophobic amino acids and γ-CyD (So *et al*., 2000; Qin *et al*., 2002; Tavornvipas *et al*., 2004). The inconsistency in the energy minimization process of free tryptophan with γ-CyD justifies the observation mentioned earlier quite reasonably. The decrease of the tryptophan fluorescence intensity with the gradual addition of γ-CyD is also notable. Remarkably, the quantum yield of tryptophan in proteins is determined by its immediate environment. Various side chains of amino

acids, as well as the peptide bond, are efficient quenchers of tryptophan fluorescence through various non-radiative processes like electron and proton transfer (Adams *et al.*, 2002). Consequently, the decrease in tryptophan emission intensity is possibly because of the changes in the immediate environment of the protein because of structural alterations in the presence of γ-CyD, as evident from CD studies (Royer, 2006).

The molecular recognition by γ-CyD is also studied with dansyl-modified Apo-Mb. The gradual addition of γ-CyD, enhances the fluorescence intensity of tryptophan with the progressive red shift of the band maxima at 355 nm (Figure 1d). The observed changes in emission spectra is in sharp contrast to the changes observed on β-CyD addition (Figure 1b and d). The emission energy of even a partially buried tryptophan invariably shifts to the red upon surface exposure (Royer, 2006), while the emission spectrum of free tryptophan in water is at 355 nm (Royer, 2006). Accordingly, the observed spectral change on γ-CyD addition signify the surface exposure of tryptophan residues (Royer *et al.*, 1993). The red shift of the band maximum further indicates the non-inclusion of the tryptophan side chain in the γ-CyD cavity (Figure 1b and d).

The complexation of Apo-Mb with CyD molecules have also been observed by monitoring the emission spectra of dansyl chromophore. Figure 3a shows the emission peak of dansyl (bound to Apo-Mb) (λ_{ex} = 320 nm) at 500 nm. The gradual addition of β-CyD progressively changes the emission peak at 484 nm with concomitant enhancement of fluorescence intensity. The blue shift of the emission maxima denotes the lowering of micropolarity around the probe, indicating the inclusion of the protein-bound dansyl molecule in the hydrophobic interior of β-CyD cavity (Scheme 1b). The significant enhancement of the dansyl fluorescence is quite similar to the enhancement of tryptophan fluorescence in β-CyD (Figure 1a and b) and can be explained because of the deactivation of non-radiative decay channels in the hydrophobic cavity of β-CyD mentioned earlier (Ghosh *et al.*, 2011). The energy-minimized structures appearing in Figure 2b also supports the recognition of dansyl by β-CyD cavity. Using dansylated lysine molecule as a model, the energy-minimized structures in Figure 2b indicates effective inclusion of the *N, N*-dimethyl naphthalene moiety of dansyl in β-CyD cavity, with estimated stabilization energy of −156 kJ mol^{-1}.

Changes in the emission spectra of the dansyl (bound to Apo-Mb) is also observed in the presence of γ-CyD molecules (Figure 3b). However, inclusion in γ-CyD produces a much smaller blue shift of the emission band maxima, with relatively little changes in the intensity in comparison with β-CyD (Figure 3a and b). The difference in the emission behavior of the dansyl molecule (bound to Apo-Mb) suggests the different binding interactions of the protein with CyD molecules of various cavity size. The smaller cavity of β-CyD allows a stronger fit of the dansyl molecule, showing significant change of the emission spectra. However, the larger cavity of γ-CyD forms loose complex with dansyl molecule, showing negligible changes in the emission spectra. The inconsistency in the energy optimization process of free dansyl-lysine moiety with γ-CyD also qualifies the observation mentioned earlier quite reasonably.

We have also applied picosecond-resolved FRET technique, between the probes tryptophan and dansyl, in order to monitor the molecular recognition of the protein by CyD molecules in a more precise way. As evident from Figure 4a, the fluorescence decay of tryptophan residues (λ_{em} = 340 nm, λ_{ex} = 293 nm) of the protein becomes faster in dansyl-modified Apo-Mb, indicating FRET from tryptophan to dansyl (Mondol *et al.*, 2012). Significantly, unlike emission in free protein (two longer components), the

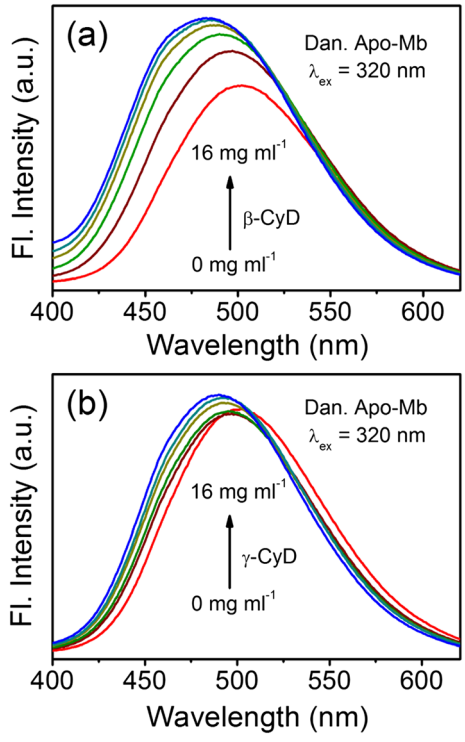


Figure 3. Steady-state emission spectra of dansyl (λ_{ex} = 320 nm) in apomyoglobin in various concentrations of (a) β-cyclodextrin, and (b) γ-cyclodextrin.

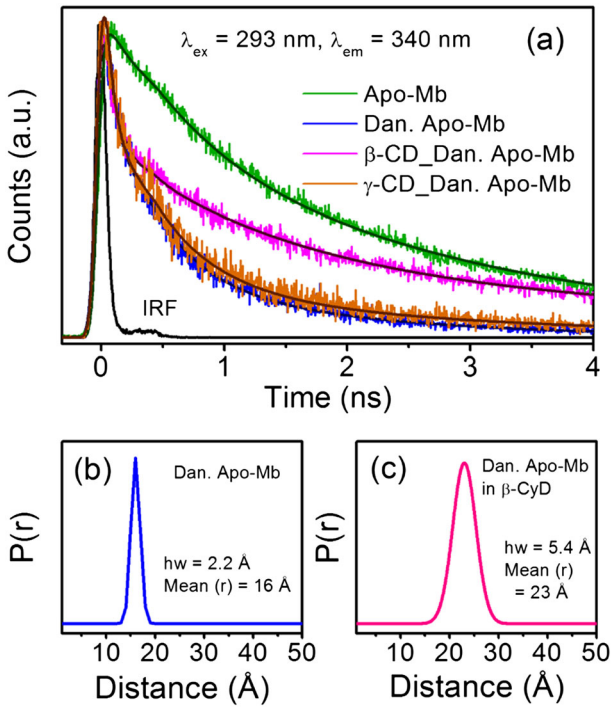


Figure 4. (a) Picosecond-resolved fluorescence decay transients of tryptophan in dansyl-modified apomyoglobin is shown in the presence of β-cyclodextrin and γ-cyclodextrin (16 mg ml^{-1}). The green line is the decay profile of tryptophan in dansyl-free apomyoglobin. (b) and (c) show the probability of distance distribution between donor and acceptor pair in absence and presence of β-cyclodextrin, respectively.

temporal decay of tryptophan in dansylated Apo-Mb shows three time components, including a faster time component of 45 ps (57%). As a consequence, the average lifetime (τ_{ave}) of tryptophan decreases from 2000 ps to 337 ps, in dansyl-modified Apo-Mb (Table 1). The Förster distance between the donor-acceptor pair is found to be 16 Å, with estimated possible distribution of hw = 2.2 Å (Figure 4b). Upon the addition of β-CyD to the dansylated Apo-Mb, the average decay time of the tryptophan emission (λ_{em} = 340 nm, λ_{ex} = 293 nm) becomes slower, compared with β-CyD free protein (Figure 4a, Table 1). The Förster distance between the donor-acceptor pair is found to be 23 Å with hw = 5.4 Å (Figure 4c). However, insignificant change in the decay profile, attributing no change in the donor-acceptor distance of the dansylated protein, is observed upon addition of γ-CyD. The result is quite consistent with the steady-state emission studies of the protein with CyDs. Stronger recognition of the protein (through tryptophan, dansyl) occurred by the smaller cavity size of β-CyD molecules. Consequently, increase in the donor-acceptor distance is realized in presence of β-CyD. However, insignificant change of the donor-acceptor distance, in presence of γ-CyD, further confirms weak complexation of γ-CyD with Apo-Mb.

The study so far discussed suggests the different molecular recognition of the protein, depending on the dimension of the host cavity. In order to establish the stoichiometric compositions of the inclusion complexes, and to determine the thermodynamic energy parameters associated with the binding, Benesi-Hildebrand method (Benesi and Hildebrand, 1949) is applied employing following equation:

$$\frac{1}{I_F - I_F^0} = \frac{1}{I_F' - I_F^0} + \frac{1}{K_i\left(I_F' - I_F^0\right)[CyD]} \quad (9)$$

Where I_F^0, I_F, and I_F' are the integrated fluorescence intensities in the absence of CyD, at intermediate and at infinite concentrations of respective CyDs and K_i being the binding constant. In β-CyD solutions, the Benesi-Hildebrand plot for tryptophan emission shows linear behavior in both the dansyl-free and dansyl-bound protein (Figure 5a and b). The linearity of the plot suggests the formation of 1 : 1 complex between tryptophan and β-CyD (Scheme 1a). From the slope and intercept of the plot, the association constant (K_i) for the inclusion complex is estimated to be 90.8 M^{-1} and 238.35 M^{-1}, for dansyl-free and dansyl-bound protein, respectively. The corresponding standard free energy changes (ΔG^o) for the complexes are found to be −11.17 kJ mol^{-1} and −13.56 kJ mol^{-1}, respectively. Significant deviation of the experimental energy value from that of the theoretical data (−108 kJ mol^{-1}) is possibly because of the consideration of free tryptophan moiety in the molecular modelling study. The hydrophobic modification of the protein surface is therefore

Table 1. Fluorescence decay parameters for apomyoglobin in presence of β- and γ-CyD

Sample Name	Cyclodextrin	a_1	τ_1/ ps	a_2	τ_2/ ps	a_3	τ_3/ ps	τ_{ave}/ ps
Apo-Mb	-	-	-	0.39	700	0.61	2840	2000
Dan. Apo-Mb	-	0.57	45	0.33	414	0.10	1691	337
Dan. Apo-Mb	γ-CyD	0.65	48	0.28	504	0.07	2621	358
Dan. Apo-Mb	β-CyD	0.58	100	0.28	1398	0.14	6862	1361

τ_i represents decay time constants and a_i its relative contribution, $\tau_{ave} = \sum a_i \tau_i$

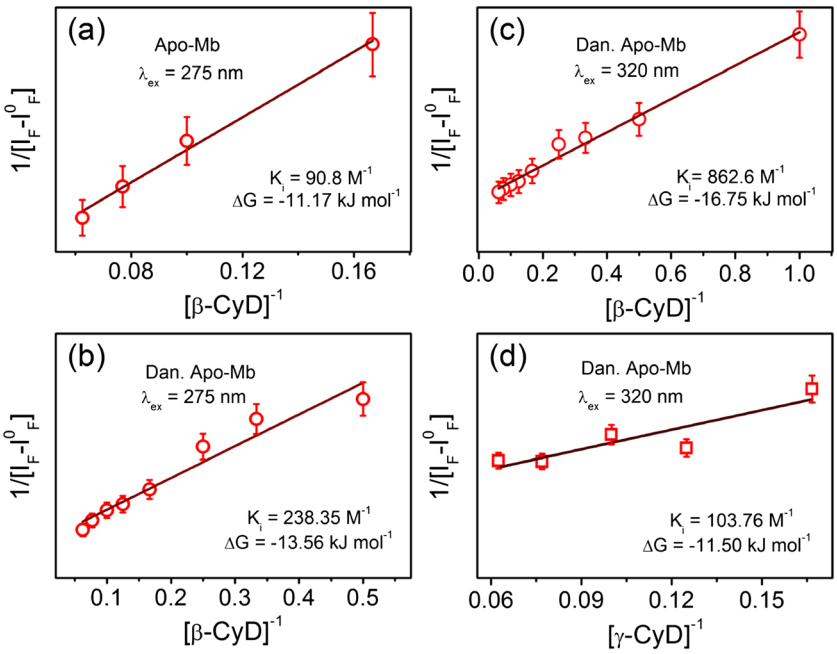


Figure 5. Benesi-Hildebrand plot for 1 : 1 complexation of tryptophan, in dansyl-free (a) and dansyl-modified (b) apomyoglobin, with β-cyclodextrin are shown. Benesi-Hildebrand plot for 1 : 1 complexation of dansyl, in apomyoglobin, with β-cyclodextrin (c) and γ-cyclodextrin (d) are shown.

found to stabilize the tryptophan inclusion of the protein into host β-CyD cavity. The dansylation of the protein initiates tighter fit of the tryptophan side chain in the β-CyD cavity, which is well reflected in the increased K_i value (by three times) and the decrease in the thermodynamic free energy value ($\Delta\Delta G^o$) of $-2.39\,kJ\,mol^{-1}$ upon dansylation. The increased recognition of the protein is probably associated with the increased localized binding site of the protein because of the conformational modification upon dansylation, discussed later.

Benesi-Hildebrand plot applying equation 9 shows linearity in protein recognition through dansyl molecule, by both the CyD molecules (Figure 5c and d). Similar to tryptophan, linearity of the plot suggests the 1:1 stoichiometry of the binding (Scheme 1b). The K_i value estimated, for β-CyD and γ-CyD association with the protein are $862.6\,M^{-1}$ and $103.76\,M^{-1}$, respectively. The corresponding ΔG^o value for the association is $-16.75\,kJ\,mol^{-1}$ and $-11.50\,kJ\,mol^{-1}$, respectively. The higher selectivity of β-CyD ($\Delta\Delta G^o = -5.25\,kJ\,mol^{-1}$) with greater K_i value of protein association is associated with the smaller cavity size of β-CyD, allowing a tighter fit of the dansyl molecule to be included. The deviation of the experimental energy value from that of the theoretical data ($-154\,kJ\,mol^{-1}$) is possibly because of the consideration of free dansyl-lysine moiety in the molecular modelling study. The estimation of K_i value for both tryptophan and dansyl association with β-CyD led us to estimate the overall equilibrium constant (K_{total}) for Dan. Apo-Mb association with β-CyD dictated by the reaction

$$\begin{aligned}\mathrm{Trp-[apo-Mb]-Dan}+2\beta\mathrm{-CyD} &\underset{}{\overset{K_{total}}{\rightleftharpoons}} (\beta\mathrm{-CyD})\\ -\mathrm{Trp-[apo-Mb]-Dan}-(\beta\mathrm{-CyD})&\end{aligned} \qquad (10)$$

This equilibrium can be split into two steps in which (β-CyD)-Trp-[Apo-Mb]-Dan and (β-CyD)-Trp-[Apo-Mb]-Dan-(β-CyD) are formed in individual steps with association constants K_1 (= $238.35\,M^{-1}$) and K_2 (= $862.6\,M^{-1}$), estimated from equation 9, respectively

$$\begin{aligned}\mathrm{Trp-[apo-Mb]-Dan}+\beta\mathrm{-CyD} &\underset{}{\overset{K_1}{\rightleftharpoons}} (\beta\mathrm{-CyD})\\ -\mathrm{Trp-[apo-Mb]-Dan}&\end{aligned} \qquad (11)$$

$$\begin{aligned}(\beta\mathrm{-CyD})-\mathrm{Trp-[apo-Mb]-Dan}+\beta\\ -\mathrm{CyD} \underset{}{\overset{K_2}{\rightleftharpoons}} (\beta\mathrm{-CyD})-\mathrm{Trp-[apo-Mb]-Dan}\\ -(\beta\mathrm{-CyD})\end{aligned} \qquad (12)$$

According to multiple equilibria model (Beck and Nagypál, 1990; Hoffmann and Nagypál, 2006), K_{total} is the product of the stepwise constants K_1 and K_2. The K_{total} value is thus estimated to be $238.35\,M^{-1} \times 862.6\,M^{-1} = 205609\,M^{-2}$, for Dan. Apo-Mb association with β-CyD.

We sought evidence of the structural changes of the protein upon CyD addition. CD spectroscopy is an efficient spectroscopic tool to study structural changes in protein. Figure 6 shows the CD structure of the protein in dansyl-free and dansyl-bound condition in the presence of various concentrations of β-CyD and γ-CyD molecules. The negative band at 208 and 222 nm is indicative of the α-helical content of the protein. In all the cases, a gradual decrease of the overall helical content of the protein is observed upon β-CyD and γ- CyD addition (Figure 6). Earlier reports suggest the solvent exposure and therefore accessibility of hydrophobic amino acid residues as a prerequisite for CyD-protein interaction (Serno *et al.*, 2011). The observed changes in tryptophan emission (Figure 1) therefore indicates the surface exposure of the tryptophan side chains on the protein surface (Scheme 1a), consistent with CD studies (Figure 6). Therefore, changes in the CD structure is quite expected. Similarly, upon dansylation, greater change of the helical content of the protein is consistent with the higher

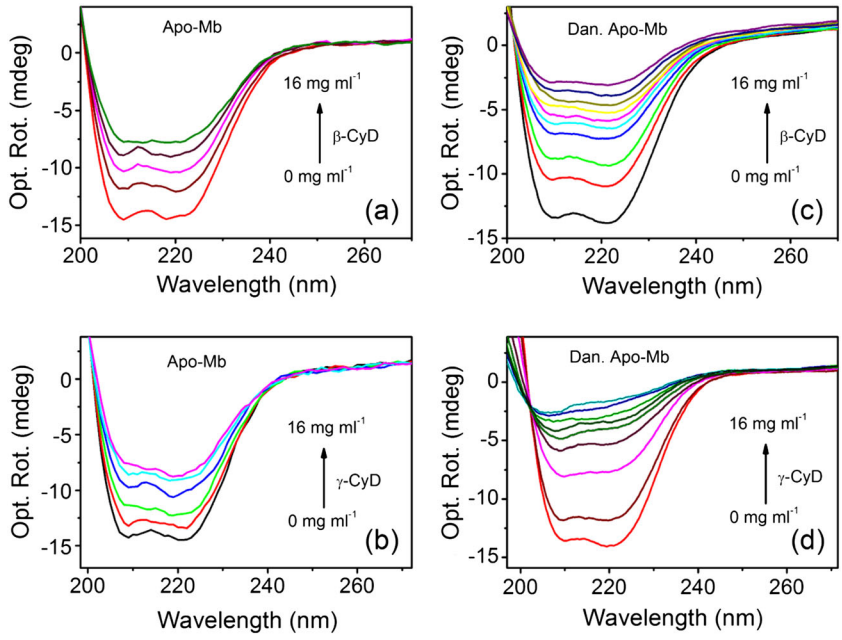


Figure 6. Circular dichroism spectra of apomyoglobin as a function of β-cyclodextrin (a) and γ-cyclodextrin (b) concentration. Circular dichroism spectra of dansylated apomyoglobin as a function of β-cyclodextrin (c) and γ-cyclodextrin (d) concentration.

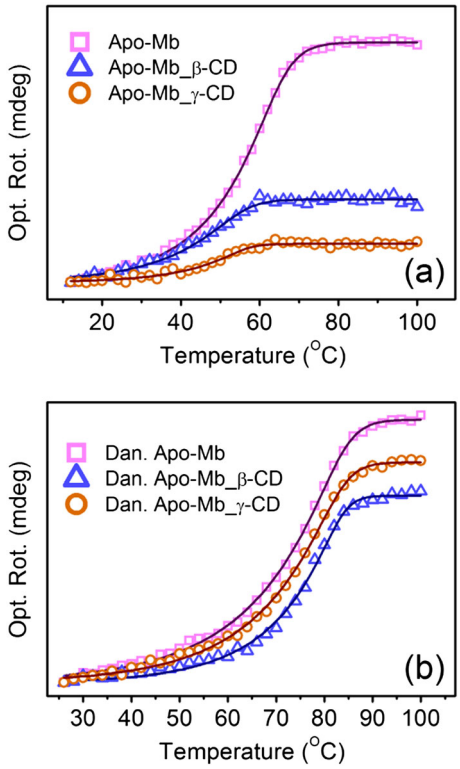


Figure 7. Temperature-dependent circular dichroism spectra of dansyl-free (a) and dansyl-modified (b) apomyoglobin, in the presence of β-cyclodextrin and γ-cyclodextrin concentration of 16 mg ml^{-1}. (All the curves were base line subtracted for comparison).

association (K_i) value of the dansylated protein in complexation with CyD (Figure 5).

We examine the thermal stability of the protein in presence of CyD by monitoring the changes in ellipticity at 222 nm. The denaturation temperature (T_m) of Apo-Mb is determined to be 56°C, as the midpoint of the transition curve depicted in Figure 7a. Significant decrease in the T_m value (about 13°C) is observed in presence of β-CyD, whereas γ-CyD shows relatively smaller effect (about 8°C) (Figure 7a). The higher magnitude of decrease in the T_m value appears to stem from the higher complexation of β-CyD compared with γ-CyD, with the protein (Figure 5). The result is quite consistent with previous observation (Tavornvipas *et al*., 2006) and clearly suggests that the binding of CyD to the exposed hydrophobic side chains of Apo-Mb during heating destabilizes the native conformations of the protein, by shifting the equilibrium in favor of the unfolded state (Cooper, 1992; Tavornvipas *et al*., 2006).

Previous report suggests that hydrophobic environment increases the thermal stability of a protein, and is the key factor for the stability of thermophilic proteins, in comparison with other factors like hydrogen bonds, and electrostatic interactions (Gromiha *et al*., 2013). The study by Georis *et al*. shows that additional aromatic interaction improves the thermostability of family 11 Xylanase (Georis *et al*., 2000). The thermal stability of Apo-Mb has also been evaluated after dansylation of the protein. Considerable enhancement of the T_m (equal to 74°C) value of the dansylated Apo-Mb is observed in comparison to the dansyl-free protein (Figure 7a and b). The observed higher thermal stability of the protein appears to stem from the higher surface hydrophobicity of the protein upon dansylation, mentioned earlier. Remarkably, protein thermal denaturation starts with unfolding of the outer surface, leading to the surface exposure of the hydrophobic core (Caflisch and Karplus, 1994). The denaturation can possibly be prevented by stabilizing the protein surface with hydrophobic interactions. The insignificant change in the thermal denaturation transition of the dansyl-modified protein, unaffected by either CyD molecules, is therefore concluded to be because of the increased hydrophobic character of protein surface upon dansylation (Figure 7b).

CONCLUSION

We have explored the effect of the attachment of a fluoroprobe at the protein surface in the molecular recognition of Apo-Mb by a synthetic receptor CyD. The significant enhancement of the CyD-protein association is observed upon the dansyl labelling of the protein. The recognition of the protein by CyD changes significantly, depending on the cavity size of the receptor. The smaller cavity of β-CyD allows steric fit of the hydrophobic residues (similar to tryptophan and dansyl) of the protein. FRET between the probes tryptophan and dansyl shows increase in the donor-acceptor distance in the presence of β-CyD. However, insignificant change of the donor-acceptor distance in presence of γ-CyD indicates a stronger association of the protein with β-CyD over γ-CyD. Molecular modelling studies on the interaction of tryptophan and dansyl probe with β-CyD is found to be consistent with the experimental observations. The unaltered melting behavior of the dansyl-attached protein by CyDs, suggests the stabilization of the protein because of hydrophobic labelling. Thus, both the cavity size of the synthetic receptor and hydrophobic character of the protein surface are found crucial in determining the molecular recognition of a protein by CyD as a synthetic receptor. The study offers a cautionary note on the use of hydrophobic fluorescent labels for proteins in biochemical studies involving the recognition of molecules.

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