Models for three DBD601 molecules binding to dsDNA

Figure 6a shows a possible model of how the DBD\textsuperscript{601} can transition from a 1:1 complex to the 2:1 and 3:1 ones. In this model there are minimally four ligated states (III, II and two singly ligated ones (Ia+Ib) and Ic) characterized by four equilibrium constants and the relative contribution to the observed signals from each bound state. Moreover, for DNA substrates containing a Rap1 recognition sequence the model is further complicated by the fact that in complex Ic and II the DBD can bind each hemi-site with higher affinity than a random site (1). Therefore, for DNA substrates containing a Rap1 recognition sequence the different affinities of the DBD for a full-site vs. a half-site vs. a random one should be considered. Because of the complexity of the system, we analyzed the equilibrium fluorescence anisotropy data with simplified models to extract preliminary estimates of some of the equilibrium constants.

We first focused on the binding of the DBD\textsuperscript{601} to the RND substrate that does not contain a Rap1 sequence. The data in Figure 3a show that the fluorescence anisotropy change of RND with FAM at the 5'-end of the top strand is sensitive to the binding of each ligand, providing an estimate of the contribution of each bound state to the observed anisotropy change. Under the assumption that the RND substrate can be considered as an homogeneous lattice (2), binding of three DBD\textsuperscript{601} molecules can be described by the non-specific ligand binding to a finite lattice as derived by Epstein (3). The partition function for the system is then
Model 1

\[ Z_e = 1 + \sum_{k=1}^{g} \sum_{j=0}^{k-1} P_M(k,j)(K_e P)^k \omega^k \]

where \( g \) is the maximum number of bound DBD\(^{601} \) molecules, \( k \) is the number of DBD\(^{601} \) molecules bound at any concentration of free protein, \( j \) is the number of cooperative contacts between bound proteins. \( P_M(k,j) \) are the statistical factors for a protein of site size \( n \) bound to a lattice of length \( M \) (3)

\[ P_M(k,j) = \frac{(M - nk + 1)! (k - 1)!}{(M - nk - k + j + 1)! (k - j)! j! (k - j - 1)!} \]

For our case in complex Ic in Figure 6a \( M=21 \), \( n=6 \) and \( P_M(1,0) = (M-n+1) = 16. \) The experimental data were fitted in Scientist (Micromath) using the expression for the degree of binding \( \nu = \frac{d\ln Z}{d\ln P} \), the equation for the conservation of mass and the relation

\[ r_{obs} = \sum_{i=0}^{g} c_i r_i \]

where \( c_i \) is the concentration of each bound state and \( r_i \) is its associated anisotropy and where any contribution of quantum yield changes to anisotropy has been neglected because of the small changes in fluorescence intensity (Figure 3a, inset). For the RND substrate from Figure 3a \( r_o = 0.076 \), \( r_1 = 0.13 \), \( r_2 = 0.189 \) and \( r_3 = 0.28 \), leaving only \( K_e \) and \( \omega \) as fitting parameters. Figure 6c shows (blue lines) the fitting with this model for the binding of DBD\(^{601} \) to RND determined at 10 nM and 255 nM DNA in Buffer HN\(_{50}\). The data at either DNA concentration are fitted well with \( K_e = 7.4 \times 10^5 \text{ M}^{-1} \) and \( \omega = 365 \).

The estimated value of the equilibrium dissociation constant of \( \sim 1 \mu\text{M} \) for the singly-ligated state is consistent with the reported 8.7 \( \mu\text{M} \) for the non-specific binding of Rap1.
determined from competition experiments using EMSA (4). Interestingly this model also suggests that binding of the second DBD molecule to a DNA of random composition would be stabilized by moderate positive cooperativity. A tacit assumption in fitting the data for RND with this model with n=6 is that complex Ia in Figure 6a does not exist. Although for DBD bound to RND both the signatures of the fluorescence anisotropy and intensity changes and the differences in the electrophoretic mobility (see Main Text) suggest that this complex is in a different conformation as compared to the one formed with DNAs containing Rap1 recognition sequence, the presence of complex Ia cannot be excluded. In order to take this into account we next considered a model where this state does exist. The partition function for this model is then (5, 6)

**Model 2**

\[
Z_m = Z_e + (M - m + 1)K_m P
\]

where \((M-m+1)\) is the statistical factor assuming that on the lattice both myb-like domains bind next to each other with twice the site-size of a single myb-like domain \((m=2n=12)\). If we were to assume that with RND complex Ia is in the same conformation as with DNAs containing a specific Rap1 sequence then \(r_m \sim 0.09\) (Figure 3a). However, in this case Model 2 does not capture the data at the higher DNA concentration (not shown). Therefore, we assumed that when bound to RND the anisotropy for complex Ia is the same as for complex Ic \((r_m=r_c=0.13)\). Figure 6c shows (red dash lines) the fitting of the anisotropy data for RND with Model 2 and \(K_m=7.5 \times 10^6\) M\(^{-1}\), \(K_c = 1 \times 10^5\) M\(^{-1}\) and \(w=8850\). Using this model the value of the equilibrium dissociation constant for complex Ia is \(~ 133\) nM, much lower than the one reported for the non-specific binding of Rap1 (4). Also, this model would suggest that binding of the second ligand is highly
cooperative and this would lead to a higher propensity to form clusters of bound proteins (7-9). It is important to point out that based solely on fitting, Model 1 and 2 cannot be discriminated. More information on the non-specific interaction of Rap1 with DNAs of random composition would be required. At this stage, Model 1 with the least number of parameters is sufficient to describe the data and it would suggest that if complex Ia exists it does not contribute significantly to the observed binding.

The situation is different for the DBD^{601} binding to DNAs that contain a centrally located 13 bp specific sequence. In this case formation of complex Ia is characterized by an equilibrium constant $K_{13}$ and the partition function is

$$Z_{13} = Z_e + K_{13}P$$

Also, in this case the data in Figure 3a show that formation of the high affinity 1:1 complex is accompanied by a small change in anisotropy $r_{13} \sim 0.09$. For the analysis of the anisotropy data with TeloA (Figure 6d) we also assumed that for complexes Ic-III the values of the anisotropy are the same as the ones observed with RND ($r_1 = 0.13$, $r_2 = 0.189$ and $r_3 = 0.28$). We initially fitted the data for TeloA using $K_e = 7.4 \times 10^5$ M$^{-1}$ and $\omega = 365$ determined for the RND substrate with Model 1 and fitting only for $K_{13}$ ($8.77 \times 10^7$ M$^{-1}$). With this strategy the model does not fit well the data at the higher DNA concentration (black line). Because of the high degree of correlation of the fitting parameters and lack of convergence $K_{13}$, $K_e$ and $\omega$ cannot be fitted simultaneously.

Therefore, we fitted Model 3 for $K_{13}$ in combination with either $K_e$ or $\omega$ keeping constant the other parameter at the value determined for RND. With fixed $K_e = 7.4 \times 10^5$ M$^{-1}$ (blue dash lines in Figure 6d) we obtain $K_{13} = 1.6 \times 10^9$ M$^{-1}$ and $\omega = 1358$. With fixed $\omega = 365$
(red dash lines in Figure 6d) we obtain $K_{13} = 5 \times 10^9 \text{M}^{-1}$ and $K_e = 2.6 \times 10^6 \text{M}^{-1}$. It is evident from Figure 6d that these two combinations cannot be discriminated. However, independent of the combination of the parameters this analysis suggests that the equilibrium dissociation constant for DBD binding to the specific site is $\sim 0.2$-0.6 nM, consistent with the reported value of 0.5 nM determined for TeloA by EMSA (10) and much higher than the affinity for a non-specific site (RND) estimated with either Model 1 or 2. Also, because of the presence of two hemi-sites and the reported ability of Rap1 to bind to a single one (1), it is reasonable to assume that complex Ic would form with higher affinity on TeloA and the fitted value of $K_e = 2.6 \times 10^6 \text{M}^{-1}$ (at fixed $\omega=365$) would suggest an increase of 3-4 fold compared to RND. Finally, independent of the combination of parameters $\omega K_e \sim 1 \times 10^9 \text{M}^{-1}$ obtained with Model 3 would suggest that even on TeloA binding of the second ligand might be stabilized by positive cooperativity. Because of the inability of Model 3 to discriminate between changes in $K_e$ and $\omega$ we next analyzed the data with an even further simplified model that assumes that while binding of DBD to the specific site occurs with binding constant $K_{13}$, binding of the DBD in complexes Ic-III occurs with the same apparent equilibrium constant $K_a$ that does not take into account either statistical factors or cooperativity.

**Model 4**

$$Z_a = 1 + K_{13}P + K_aP + K_a^2P^2 + K_a^3P^3$$

For this model we used the same $r_1, r_2$ and $r_3$ as for Model 3 and fitted the data for $K_{13}$, $K_a$ and $r_{13}$. Model 4 fits the TeloA anisotropy data in Figure 6d with $r_{13} = 0.086$, $K_{13} = 2.9 \times 10^9 \text{M}^{-1}$ and $K_a = 1.4 \times 10^8 \text{M}^{-1}$. This analysis shows that the equilibrium dissociation constant for DBD binding to the specific site ($\sim0.3$ nM) is essentially the same as the one
determined with Model 3, suggesting that this parameter is the best defined. The value of $K_a = 1.4 \times 10^8 \text{M}^{-1}$ is however 3-12 times higher than the ones obtained from $(M-n+1)*K_e$ with Model 3 ($4.2 \times 10^7 \text{M}^{-1}$ or $1.2 \times 10^7 \text{M}^{-1}$), suggesting that if in complex Ic the DBD binds preferentially to one of the hemi-sites (1) it might do so with higher affinity than expected based on Model 3. Also, the $K_a$ estimated for TeloA with Model 4 is 12 times higher than $(M-n+1)*K_e = 1.2 \times 10^7 \text{M}^{-1}$ estimated for RND with Model 1, suggesting that indeed the DBD in complex Ic has higher preference for the centrally located recognition sequence. Finally, similar to the RND data also for TeloA the models cannot be discriminated solely on the basis of the fitting and the precise determination of the different equilibrium constants will require additional information on the system.

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