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## Analysis of the DNA-Binding Properties of MyoD, Myogenin, and E12 by Fluorescence Anisotropy<sup>†</sup>

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ABSTRACT: MyoD and Myogenin are dominant myogenic regulatory factors (MRFs), which are involved in control of muscle-specific gene expression. The ubiquitously expressed E12 dimerizes with MyoD and Myogenin and has been shown to enhance their DNA-binding and transcriptional activities. In this study, fluorescence anisotropy assays have been used to determine the Gibb's free energy of dissociation ( $\Delta G$ ) for MyoD, Myogenin, and E12 as homo- and heterodimers to the well-characterized myosin light chain enhancer (MLC), muscle creatine kinase (MCK) enhancer, and mutant thereof. The heterodimers of MyoD or Myogenin with E12 bound the MCK enhancer equally well ( $\Delta G = 21 \text{ kcal/mol}$ ). The homodimers varied dramatically in both MLC and MCK enhancer binding affinity. MyoD homodimer bound the MCK enhancer with the highest affinity ( $\Delta G = 19.6 \text{ kcal/mol}$ ) in comparison with the Myogenin homodimer— MCK interaction ( $\Delta G = 16.6 \text{ kcal/mol}$ ) and E12 homodimer-MCK interaction ( $\Delta G = 18.0 \text{ kcal/mol}$ ). The slope and shape of the binding isotherms revealed that with the exception of the E12 homodimer— MCK enhancer interaction, the other proteins bound with high levels of positive cooperativity. In contrast, the E12 homodimer-MCK enhancer interaction actually occurs with significant negative cooperativity. The binding of these proteins to MLC enhancer mimicked binding to the MCK enhancer, but with much lower affinities. These data support the hypothesis that DNA acts as an allosteric ligand facilitating the dimerization of these proteins. The combination of differential affinity and cooperativity explains why the heterodimers are the active species in transcriptional regulation.

Vertebrate development results from the highly coordinated, temporal control of gene expression in response to the action of a large number of interacting transcription factors. Myogenesis is one of the best understood of the developmental pathways, and many of the regulatory factors have been identified and characterized (reviewed in refs *I* and 2). The myogenic regulatory factors (MRFs),<sup>1</sup> which were shown to be responsible for the muscle phenotype, are members of the superfamily of basic helix—loop—helix (bHLH) transcription factors that regulate cell-lineage-specific transcription and proliferation. MyoD, Myogenin, Myf5, and MRF4, which comprise the myogenic helix—loop—helix (mHLH) family, can activate the myogenic program in nonmuscle cells when ectopically expressed.

The crystal structure of the bHLH domain of MyoD dimer bound to DNA has been solved (3). The basic domain is involved in sequence-specific DNA binding and the HLH motif mediates dimerization with other HLH containing proteins (4) such as E12, E47, HEB, Id, and twist (5-8). E12 and E47 are alternatively spliced, ubiquitous products of the E2A gene, which are thought to enhance the activity of the myogenic proteins in vivo (9, 10) and in vitro (7, 11, 12) through heterodimerization and subsequent DNA binding.

The association of various transcription factor subunits into a functional regulator is commonplace in eukaryotes, in comparison with the paradigmatic regulators in bacteria which are relatively stable oligomers. In addition to the myogenic proteins described above, the Myc/Max bHLH regulators (reviewed in 13) and the nuclear hormone receptors utilize combinatorial mechanisms to achieve their regulatory goals (14). In our initial study of the myogenic regulatory factors, we found that an accurate description of the equilibrium binding constants for the complexes formed was not possible because of this combinatorial mechanism (15). Complicating the analysis further was the presence of multiple DNA binding sites and differential levels of cooperativity between the bound regulatory proteins. In an effort to deconvolute these complex mechanisms, we set out to define the individual protein-protein interactions involved, in that case, MyoD and E12. We found that MyoD ho-

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<sup>&</sup>lt;sup>1</sup> Abbreviations: MCK, muscle-specific creatine kinase; MLC, myosin light chain; MRF, myogenic regulatory factor; HLH, helix-loop-helix; bHLH, basic helix-loop-helix.

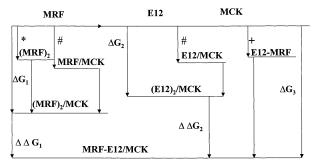


FIGURE 1: Thermodynamic linkage diagram of the specific interactions between muscle regulatory factors (MRF), E12, and the MCK enhancer DNA.  $\Delta G_1$  is the free energy of the MCK enhancer bound by the MRF homodimer,  $\Delta G_2$  is the free energy of the MCK enhancer bound by the E12 homodimer, and  $\Delta G_3$  is the free energy of the MCK enhancer bound by the MRF/E12 heterodimer, all starting from the free monomer species plus free enhancer DNA.  $\Delta\Delta G_1$  is the difference free energy of the MCK bound by the MRF homodimer with respect to the E12/MRF heterodimer, and  $\Delta\Delta G_2$ is the difference free energy of the MCK bound by the E12 homodimer with respect to the E12/MRF heterodimer. The free energies of homo (\*) and hetero (+)-dimerization previously reported in ref 16 and used as fixed parameters in data analysis. The # symbols refer to the free energies of the monomer bound MCK. These values are recovered from the homodimer/MCK binding data but, except in the case of the E12/MCK interactions, are not well determined due to high levels of cooperativity.

modimers and MyoD-E12 heterodimers were equally stable in solution, in the absence of DNA (16). Given the relatively low stabilities of these complexes, MyoD and E12 are likely to be monomeric in the cell, when not bound to DNA. In this report, we have taken the analysis to the level of multiple proteins and their interactions with DNA. We have applied the solution-based, equilibrium binding assay fluorescence anisotropy to determine the free energies describing the dissociation of the relevant complexes. The data have been fit to a model that includes all possible species (Figure 1). The results indicate that differential cooperativity is key to the formation of the active regulators and bias their formation over other complexes. In agreement with the work from others, DNA appears to act as an allosteric ligand and enhances the binding of the appropriate second subunit of the protein. Taken together, our results support the widely held notion that preferential heterodimerization of the MRFs with an E-protein is responsible for driving muscle-specific gene expression during myogenesis but that the heterodimers are DNA induced rather than forming off DNA.

### MATERIALS AND METHODS

MyoD, Myogenin, and E12 proteins. Full-length MyoD, Myogenin, and E12 proteins were overexpressed in Escherichia coli strain BL21(DE3)pLysS (Novagen) from T7 expression plasmids and highly purified as described (17). The proteins were judged to be approximately 95% pure following two-dimensional gel electrophoresis and Coomassie Brilliant Blue staining. Small aliquots of purified protein were stored at -80 °C. For all experiments concentrated frozen samples were thawed on ice and desalted into SJM buffer pH 7.6 (100 mM NaCl, 5 mM DTT, 1 mM EDTA, 10 mM HEPES, pH 7.9, and 5% glycerol) using disposable, calibrated gel filtration columns (10DG, BioRad). The concentrations of MyoD, Myogenin, and E12 were determined by absorbance at 280 nm using extinction

coefficients of 17 750, 21 980 M<sup>-1</sup>, and 17 900 M<sup>-1</sup>, respectively, which were calculated using the method of Gill and von Hippel (18).

Fluorescein-labeled DNA. The oligonucleotides were synthesized by the Molecular Biology Core facility at University of Arkansas Medical Sciences (UAMS) with a commercially obtained 5'-fluorescein phosphoramidite-label (Peninsula Laboratories). The oligonucleotides used are shown below demonstrating the position of the fluorescein label at the 5' end. The E-box (CANNTG, in which the N's can be any base) in each DNA is underlined. Complementary unlabeled oligonucleotides were used to generate the double stranded targets for binding analysis.

### 18 base pair MCK-right enhancer: 5'-fluorescein—CCCCAACACCTGCTGCCTT

MCK enhancer half site mutant is identical to MCK-right enhancer above except for E-box mutations shown below. We have also used the myosin light-chain enhancer sequence which is identical to the MCK enhancer in terms of E-box sequence but which contains different flanking sequences.

### 20 base pair MLC enhancer: 5'-fluorescein—ATTTTGCACCTGGCTGCTAT

We have also probed the role of dimerization by monitoring binding to a sequence derived from the MCK enhancer, but which contains three additional base pairs inserted between the two NN nucleotides of the CANNTG E-box sequence. These additional bases are in lowercase letters with the E-box underlined (only top strand is shown for simplicity):

### mutant 3 (mut3):

### 5'-fluorescein-CCCAACACgggCTGCTGCCT

The fluorescein-labeled oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis (PAGE, 7 M Urea, 1X TBE), followed by elution, phenol extraction, and ethanol precipitation. Oligonucleotide concentrations were calculated from the measured absorbances at 260 nm. Complementary oligonucleotides were synthesized, gel purified, and annealed by combining equimolar amounts of each (in 10 mM Tris-Cl pH 7.5, 1 mM MgCl₂), heating to 95 °C for 10 min and cooling to room temperature slowly in the heating block. Annealed oligonucleotides were stored in small aliquots at −20 °C and thawed freshly as needed.

Fluorescence anisotropy. This assay has been described in detail (I6, I9, 20). In brief, measurement of fluorescence anisotropy reveals the average angular displacement of the fluorophor, which is dependent on the rate and extent of rotational diffusion. An increase in the size of the macromolecule through complex formation results in decreased rotational diffusion of the labeled species, which in turn results in an increase in anisotropy. Using parallel polarized excitation, the anisotropy of a sample is calculated as the ratio of the difference between the parallel ( $I_{\parallel}$ ) and perpendicular ( $I_{\perp}$ ) emitted light and the total intensity ( $I_{\rm t} = I_{\parallel} + 2I_{\perp}$ ):

$$A = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp}) = (I_{||} - I_{\perp})/I_{\rm t}$$

Anisotropy measurements. All fluorescence measurements were made using a Beacon fluorescence polarization spec-

trometer (Pan Vera, Madison, WI) with fixed excitation (490 nM) and emission (530 nM) wavelengths. All fluorescence measurements were taken at room temperature (24 °C) in SJM buffer (see above) in a final volume of 1.1 mL. The fluorescein-labeled, annealed enhancer DNAs were diluted with SJM buffer and mixed with various concentrations of MyoD, Myogenin, and E12 as homodimers or heterodimers. Serial dilutions (by 0.5 or 0.8 vol/vol increments) of the proteins were made in SJM buffer, and the appropriate amounts were added to constant amounts of fluorescein-DNA. All heterodimer titrations were done with equimolar amounts of each protein unless indicated otherwise. In the cross titrations of the heterodimers (Figures 3 and 4), the concentrations of the DNA as well as one of the proteins were held constant, and various amounts of a second protein were mixed with them. For example, to solutions of 1 nM of fluorescein-labeled DNA and 10 nM MyoD in several tubes were added different concentrations of E12. The samples were mixed and allowed to equilibrate for 30 min at room temperature before anisotropy measurements were taken. Corresponding blanks containing everything in the above samples except the fluorescein-DNA were subtracted for each sample. The intensity of fluorescence remained constant throughout the anisotropy measurements.

Analysis of anisotropy data. Crystallographic (3), EMSA (e.g., 9, 10, 21-25), and cross-linking (16) data indicate that these proteins interact with DNA as dimers. The anisotropy of MyoD, Myogenin, and E12 binding to the fluorescent enhancer DNAs have been plotted on the ordinate as millianisotropy (mA in arbitrary units, au) versus the concentrations of the monomer of these proteins on the abscissa. Each experiment was performed on at least three independent occasions. To describe the linked equilibria involved in MyoD, MyoG, and E12, E-box interactions we utilized a numerically based algorithm (BIOEQS) which solves directly for the species populations based on the values of the  $\Delta G$ for the dissociation of the complexes to the free elements (described in detail in refs 16, 19, 20). The values of  $\Delta G$  of dissociation are positive, and the higher the value the higher, the probability that the reaction will occur. The model used was that shown in Figure 1 and described in detail in the Results section. For the MyoD homodimer titrations, the value of dimerization free energy was fixed at a value of 8.7 kcal/mol from our previous study (16). In that study the Myogenin dimerization free energy could not be determined due to solubility constraints (Maleki, S. J., Royer, C. A., and Hurlburt, B. K., unpublished data). Since the HLH domain responsible for the majority of dimerization energy of MyoD and Myogenin are homologous, we used the same free energy value (8.7 kcal/mol) in fitting the Myogenin homodimer titrations.

In the case of the cross titrations, in which one of the protein concentrations was fixed and only the other was varied, the values of the free energies for the dissociation of the homodimer/DNA complexes were fixed at those recovered from the fits of the data in Figure 2. Likewise, the MyoD and Myogenin dimerization free energies were fixed in the fit; however, the dimerization energy of E12, which has not been shown to form below  $20~\mu M$  (16), was not considered. The values of the putative monomer—DNA complexes were arbitrarily fixed at a point halfway between that of the free DNA and the dimeric complex. Since these complexes were

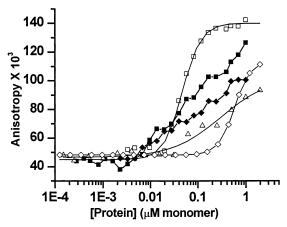


FIGURE 2: Binding isotherms of the MyoD family proteins on the MCK enhancer. Titration of 1 nM fluorescently labeled MCK enhancer with MyoD ( $\square$ ), Myogenin ( $\diamondsuit$ ), E12 ( $\triangle$ ), equimolar MyoD-E12 ( $\blacksquare$ ), and equimolar Myogenin-E12 ( $\spadesuit$ ). The titrations of homodimer binding to the MCK enhancer DNA were fit using BIOEQS and the fits are shown. The titrations using mixtures of MRF protein and E12 could not be fit and are shown as line and symbol.

not populated to a significant extent due to the high level of cooperative binding of the second monomer, these values did not noticeably affect the recovered free energies for the heterodimer-DNA dissociation. The plateau anisotropy values for the homodimer/MCK enhancer complexes were also fixed in the fit to the values recovered from the fits in Figure 2. The low and high level plateau values and the free energy values for the heterodimer/MCK enhancer complex were the only parameters floated in these fits. Uncertainties on the recovered free energy values were determined by rigorous confidence limit testing. This involves refitting the data at each of the values tested for a given parameter. Typically parameters are tested at  $\pm 50\%$  of their value in 0.2-0.5% steps. Such rigorous confidence limit testing takes into account all of the parameter correlations since all the other parameters in the fit are allowed to float during these tests. Thus, the rigorous confidence limits provided are those which truly take into account the correlations between parameters. In highly cooperative systems, often the  $\Delta G_1$  is not well resolved, and we can only place upper limits on its value. Typically, the uncertainty associated with the recovered parameters is larger when one carries out this rigorous confidence limit testing than when one only takes into account the diagonal elements of the correlation matrix.

#### RESULTS

Binding of MyoD, Myogenin, and E12 to the MCK enhancer. Many enhancers contain more than one E-box, which allows multiple levels of allostery and cooperativity for the regulatory proteins that bind. The design of experiments and the subsequent data analysis that would yield unambiguous conclusions necessitated simplification of the multiple equilibria involved; therefore, we chose to study the proteins interacting with an oligonucleotide bearing a single E-box sequence. In Figure 1 is shown a linkage diagram depicting the complexity that exists even with a single binding site. The model includes free DNA and monomeric proteins, as well as monomer—monomer, monomer—DNA, and homo- and heterodimer—DNA interactions. The free energies ( $\Delta G_1$ ,  $\Delta G_2$ , and  $\Delta G_3$ ) in the diagram in

Table 1: Results for MyoD, Myogenin, and E12 Binding to MCK Enhancer DNA

protein	DNA	$\Delta G_2$ (kcal/mol)	$\Delta G_1$ (kcal/mol)	$\Delta G_{ m coop}$ (kcal/mol) <sup>a</sup>
2 MyoD	MCK	19.6 (±0.5)	0.49	>2.5
2 E12	MCK	$18.0 (\pm 0.5)$	9.6	-1.2
2 Myogenin	MCK	$16.6 (\pm 0.3)$	6.2	>2.5
$MyoD/E12^b$	MCK	$21.0 (\pm 0.6)$	na	1.4, 3.0
$E12/MyoD^b$	MCK	20.7 (+0.2/-0.6)	na	1.1, 2.7
Myogenin/E12b	MCK	$20.8 (\pm 0.4)$	na	4.2, 2.8
E12/Myogenin <sup>b</sup>	MCK	$21.1 (\pm 0.3)$	na	4.5, 3.1

 $^a$  For the crosstitrations there are two  $\Delta G_{\rm coop}$  values. The first values describes the increase in free energy relative to the MRF homodimer alone, and the second value describes the increase in free energy relative to E12 homodimer. For example, the MyoD/E12 value of 21 kcal/mol is 1.4 kcal/mol better than the 19.6 kcal/mol value for MyoD homodimer and 3.0 kcal/mol better than the 18.0 kcal/mol value for the E12 homodimer.  $^b$  The titrant protein in cross-titration experiments.

Figure 1 correspond to free energies of dissociation of the MRF homodimer/enhancer DNA complex, the E12 homodimer/enhancer DNA complex, and the heterodimeric MRF/E12/Enhancer DNA complex, respectively, starting from the free monomeric and free DNA species. The difference in free energies ( $\Delta\Delta G_1$  and  $\Delta\Delta G_2$ ) correspond to the difference in affinity observed for the binding of the heterodimer to the enhancer with respect to the MRF and E12 homodimers, respectively. In Figure 2 are shown the binding isotherms of MyoD, Myogenin, and E12, as individual proteins (open symbols), as well as equimolar mixtures of MyoD-E12 and Myogenin-E12 (closed symbols) to 1.5 nM fluorescein-labeled MCK enhancer. In the case of the MyoD, E12, and Myogenin alone, the binding isotherms were analyzed using the biological equation solver algorithm, BIOEOS. The analysis model (Figure 1) included a free energy for the monomer-DNA interactions, the homodimer-DNA interactions, and the heterodimer/DNA interactions. The model also includes a free energy of dissociation of the homodimers (\*) and the heterodimer (+) in the absence of DNA. The value of the free energy for MyoD homodimerization has been determined previously by Maleki et al. (16) to be 8.7 kcal/mol and was therefore fixed at this value in the fits. The homodimer affinity of Myogenin was assumed to be similar to that of MyoD because of the homologous HLH dimerization domain and was therefore also fixed at 8.7 kcal/mol. No homodimerization of E12 monomers was observed in our previous studies (16) up to a concentration of 20 micromolar, and thus this species was not included in the model.

The Gibbs free energies of dissociation ( $\Delta G$ ) resulting from fits of the data in Figure 2 for the dimer and the monomer/DNA species for MyoD, E12, and Myogenin homodimer—MCK enhancer interactions are given in Table 1. The cooperative free energy value is calculated as the difference between the free energy of the dimer/DNA complex and that of the monomer/DNA complex ( $\Delta G_{\rm d} - \Delta G_{\rm m}$ ) and corresponds to how much better the second monomer binds with respect to the first. However, an equilibrium titration cannot distinguish between the intermediate population of a monomer-bound DNA species or of an intermediate free dimer, and in fact both could be populated at some level in our studies. The cooperative free energy values simply provide an idea of the extent to which the reaction is coupled or cooperative. Comparing the free

energies of the dimer—DNA species, we observe that the MyoD homodimer bound the MCK enhancer with considerably higher affinity than Myogenin. The MyoD—MCK interaction was also highly cooperative. Unexpectedly, the E12 homodimer bound this DNA target with higher affinity, but lower cooperativity than Myogenin.

Due to the fact that the titrations with equimolar mixtures of E12 with Myogenin or MyoD (Figure 2, closed symbols) involve changing the concentration of 2 elements (i.e., MyoD and E12) at the same time, the binding isotherms could not be fit for apparent affinities. In fact, since these titrations represent a diagonal in an x-y plane in which x is the concentration of MyoD, for example, and y that of E12, the number of species, free homodimers, and heterodimers, both monomer-bound DNA species, and homodimeric and heterodimeric DNA bound species were too numerous for their relative stabilities to be resolved from the data, even though theoretically BIOEQS can handle such complexity. Nonetheless, comparison of the isotherms for the equimolar mixture of MyoD and E12 with those of the homodimers reveals that some binding occurs at concentrations lower than that observed for MyoD homodimer, indicative of enhanced binding by the MyoD/E12 heterodimer. However, given that the high concentration region of the curve is quite similar in aspect to that of E12 homodimer binding, we cannot rule out binding by a mixture of homo and heterodimers in these experiments. In contrast, for the titration with equimolar mixtures of Myogenin and E12, significantly enhanced affinity was observed compared to the case of the homodimers. The cooperativity of binding for the equimolar mixture is nonetheless much lower than that observed for the Myogenin alone, indicating that more than one type of species may be formed in this case as well. The MyoD and Myogenin homodimers bound the MCK enhancer with significant positive cooperativity, whereas the E12 homodimer bound with negative cooperativity. These results suggest that the bias in forming heterodimer complexes upon DNA may be derived from differential, DNA-induced cooperativity.

Cross titrations of heterodimers. Due the complications in data analysis described above for equimolar titrations of the MCK enhancer, we performed a series of what we termed "cross titrations", in which there is only one titrant protein. Four additional titrations were performed to reveal these interactions more clearly. In Figure 3 is shown the anisotropy profile in which a constant amount of fluorescein-labeled MCK enhancer DNA (1.5 nM) and Myogenin (10 nM, a concentration at which the Myogenin homodimer does not interact with the DNA, see Figure 2) were titrated with E12. The reciprocal experiment was also performed where E12 concentration was held constant (20 nM), and the concentration of Myogenin was varied (also in Figure 3). Analogous cross titrations were performed for MyoD (held constant at 10 nM) and E12 (Figure 4). The binding data in Figures 3 and 4 were analyzed with BIOEQS using the model shown in Figure 1. The free energy of binding of the homodimers of MyoD, Myogenin, and E12 to DNA (19.6, 18.0, and 16.6 kcal/mol from analysis of the data in Figure 2) and the binding of these monomers to the DNA (also from analysis of the data in Figure 2, values in Table 1) were fixed in the fit. The plateau anisotropy values for the homodimeric/MCK complexes were also fixed in the fit to the values recovered

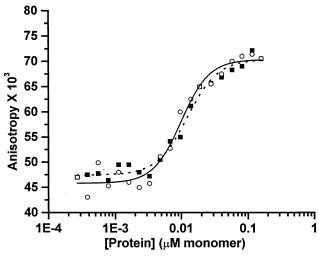


FIGURE 3: Binding isotherm of cross titration measurements for Myogenin and E12 heterodimers binding to the MCK enhancer. Titration of 1 nM MCK enhancer and 10 nM Myogenin with E12 (O). Titration of 1 nM MCK enhancer and 20 nM E12 with Myogenin (■). The data were fit with BIOEQS. The solid line is the fit for E12 as the titrant protein. The dashed line is the fit with Myogenin as the titrant protein.

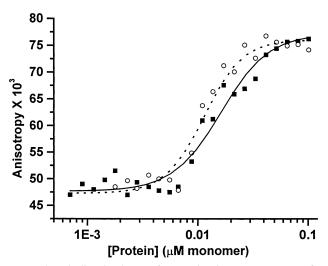


FIGURE 4: Binding isotherm of cross titration measurements for MyoD and E12 heterodimers binding to the MCK enhancer. Titration of 1 nM MCK enhancer and 10 nM MyoD with E12 (○). Titration of 1 nM MCK enhancer and 20 nM E12 with MyoD (■). The data were fit with BIOEQS. The solid line is the fit for MyoD as the titrant protein. The dashed line is the fit with E12 as the titrant protein.

from the data in Figure 2. However, the plateau value for the heterodimer complex was allowed to float. The  $\Delta G$ values for the heterodimer/MCK enhancer complex and for the difference in free energy with respect to the binding of the corresponding homodimers to this oligonucleotide are given in Table 1, where the titrant protein is indicated with b. MyoD and Myogenin heterodimers with E12 bound the MCK enhancer element with equal affinity  $\Delta G \sim 21$  kcal/ mol. It should be noted that the affinity of the Myogenin-E12 heterodimer for the MCK enhancer was significantly higher than that of either the Myogenin or E12 homodimers. Indeed, in the last two lines and last column of Table 1, it can be seen that the heterodimer of Myogenin/E12 binds to the MCK enhancer with over 4 kcal/mol more energy than does the Myogenin homodimer alone. Either of the heterodimers (MyoD/E12 or Myogenin/E12) also bound to the

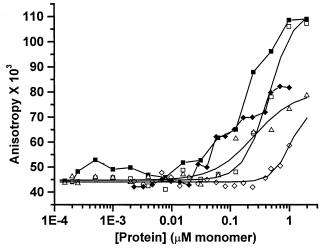


FIGURE 5: Binding isotherm of MyoD, Myogenin, and E12 homoand heterodimers with the MLC enhancer. Titration of 1 nM fluorescently labeled MLC with MyoD (□), Myogenin (⋄), and E12 (△), equimolar MyoD-E12 (■), and equimolar Myogenin-E12 (◆). The data for the homodimers (open symbols) were fit with BIOEQS. The data for the equimolar mixtures of MRF and E12 (solid symbols) were not fit and shown as line and symbol.

Table 2: Results of the Analysis of the Homodimer Binding to the Variant Target Sequences

protein	target DNA	$\Delta G_2$ (kcal/mol)	$\Delta G_1$ (kcal/mol)	$\Delta G_{ m coop}$ (kcal/mol)
MyoD	MLC	17.1 (±0.2)	5.4	>2.5
Myogenin	MLC	<16.5	< 0.2	nd
E12	MLC	<18.9	8.5	nd
MyoD	Mut3	$17.4 (\pm 0.2)$	7.1	< 2.5
Myogenin	Mut3	$15.9 (\pm 0.2/-0.7)$	7.2	1.5
E12	Mut3	15.9 (+0.5/-1.0)	< 0.2	nd

MCK enhancer with about 3 kcal/mol more affinity than did the E12 homodimer. The enhancement due to heterodimerization was more modest (a bit more than 1 kcal/mol) with respect to the MyoD homodimer, since the affinity of the MyoD homodimer was already quite high (19.6 kcal/mol).

Binding of MyoD and E12 homodimers to the MLC enhancer and an MCK enhancer mutant. The structure of the MyoD bHLH domain bound to DNA has been solved (7). The naturally occurring E-box contains two half sites with dyad symmetry CANNTG. Residues in the basic region of MyoD make phosphate and specific base contacts with the conserved CA and TG of the E-box. Each monomer makes specific contacts with one-half of the palindromic E-box. However, the overall affinity may involve interactions outside the E-box. Therefore, we have measured binding of the three homodimers and equimolar mixtures of E12 with either MyoD or Myogenin to the myosin light chain enhancer which exhibits the same E-box sequence but very different flanking sequences (see Material and Methods section). The anisotropy profiles of these titrations can be seen in Figure 5. The homodimer binding profiles (open symbols) were fit, as for the MCK enhancer using BIOEOS using the model described in Figure 1, and the recovered free energy values are given in Table 2. All three homodimers exhibited a lower overall affinity for this sequence compared to that for the MCK enhancer, although only the free energy of the MyoD homodimer/MLC complex was well determined. This is due to the fact that the lower affinity precluded complete titrations

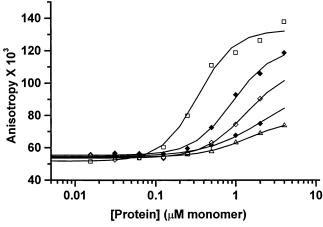


FIGURE 6: Binding isothrem of MyoD, Myogenin, and E12 homoand heterodimers with the MCK mut3 enhancer. Titration of 1 nM mut3 DNA with MyoD (□), Myogenin (⋄), and E12 (△), equimolar MyoD-E12 (♠), and equimolar Myogenin-E12 (■). The data for the homodimers (open symbols) were fit with BIOEQS. The data for the equimolar mixtures of MRF and E12 (solid symbols) were not fit and shown as line and symbol.

given the protein concentration of the samples that we were able to obtain. The decrease in affinity for this sequence highlights the fact that either DNA structure or protein contacts outside of the E-box can contribute to the overall affinity. Comparing the titrations obtained with equimolar mixtures of either Myogenin or MyoD with E12 (closed symbols) to those obtained for the homodimers, the enhanced binding of the heterodimers is quite obvious. Thus, despite the overall loss in affinity for this sequence, the DNA directed enhancement of heterodimer binding is still operative.

To examine the contributions of DNA-induced cooperativity to the ternary complex, we chose to test binding to a mutant DNA target in which the critical CA and TG half sites are separated by the insertion of three extra bases, mut3 (CACgggCTG). This target was titrated with the combinations of homodimeric (open symbols) and heterodimeric (closed symbols) proteins described above (Figure 6). The data for the homodimer-DNA interactions were analyzed by BIOEQS using the model shown in Figure 1, and the free energies for the homodimer interactions are given in Table 2. Since equimolar amounts of the proteins were used in the heterodimer binding experiments, as opposed to cross titrations, the data could not be accurately analyzed by BIOEQS. Cross titrations were not feasible because of the low-affinity binding to the mutant DNAs. Although a large decrease in affinity was observed for the MyoD homodimer binding to the mut3 sequence in comparison with binding the wild-type MCK enhancer, the high degree of cooperativity indicates that the ternary complex of the MyoD formed involves strong protein-protein interactions. The Myogenin/ mut3 interaction was reasonably well determined and found to be of slightly lower affinity than that for the MCK complex, whereas the E12/mut3 interaction was of significantly lower affinity. In contrast, while the MLC target was competent for enhanced heterodimer binding, the mut3 target was not. The titrations involving the equimolar E12:MyoD or Myogenin mixtures were found to be halfway between the two corresponding homodimeric titrations, indicating that this mutated target abolishes the DNA induced proteinprotein interactions in the heterodimers due to the incorrect spacing (and in three dimensions orientation) of the half-sites. It should be noted that the same titrations done for mut3 were performed with another DNA target we called mutR in which one-half of the E box binding site was reversed (e.g., CAnnTG → CAnnGT). The binding to this DNA was indistinguishable from the mut3 results (data not shown).

#### DISCUSSION

Myogenesis involves a complex set of interactions from a signal transduction cascade to specific alterations in gene expression involving a complex interplay of a number of transcription factors. In the end, myogenic regulatory factors (MRFs) activate a set of genes responsible for the formation of muscle. Among the many factors shown to be important for myogenesis, the MyoD family of transcriptional regulators plays a dominant role. Due to the wealth of functional, biochemical, and structural information about the MyoD family members, we have chosen them as a model for dissecting the combinatorial mechanism by which eukaryotic transcription factors control specific gene expression. The MyoD family consists of four known members: MyoD, Myogenin, MRF4, and Myf-5, all of which can convert a nonmuscle cell to the myogenic lineage when expressed ectopically. These proteins also exhibit striking homology, particularly in the bHLH domain. The MRFs bind to ubiquitously expressed products of the E2A gene, e.g., E12 and E47.

In this study, we have characterized the interactions of MyoD, Myogenin, and E12 with the muscle-specific creatine kinase gene enhancer's right E-box. Purified, full-length proteins were analyzed using fluorescence anisotropy for E-box binding as homodimers and heterodimers. The thermodynamic linkage model we applied to this analysis (Figure 1) describes all of the specific interactions between these proteins and the target DNA. We found that MyoD-MyoD bound the DNA with the highest affinity of the homodimers, followed by E12-E12 and Myogenin-Myogenin. Whereas MyoD and Myogenin homodimers bound the E-box with considerable positive cooperativity, E12 homodimers actually bound with negative cooperativity. Previous findings and this result indicate that E12 likely binds the E-box by a mechanism involving the initial formation of an E12 monomer-DNA complex, followed by association of a second E12 monomer to generate the ultimate E12-E12-DNA complex. Therefore, the simplest interpretation of the negative cooperativity in the formation of the E12-E12-DNA complex is that a E12 monomer—DNA complex is set to accept either MyoD or Myogenin, rather than a second monomer of E12. On the other hand, both MyoD homodimers and Myogenin homodimers are much more stable than either monomer-DNA complex. Thus, these MRFs, from a thermodynamic perspective, may bind DNA as preformed dimers, rather than binding sequentially as monomers. These equilibrium titrations cannot distinguish between the two mechanisms.

The most interesting conclusions of this study come from consideration of the mechanism for formation of MRF–E12–DNA heterodimeric complexes. We have previously shown that MyoD–MyoD homodimers and MyoD–E12

heterodimers are equally stable in solution in the absence of DNA (16), with  $\Delta G$ s of dissociation of 8.7 kcal/mol. However, the E12 monomer—DNA complex is more stable than those complexes by approximately 1 kcal/mol. Using these values the thermodynamically most favorable mechanism for the formation of the MRF-E12-DNA complex is one in which E12 binds the E-box first. This dimeric complex of E12-DNA is then bound by a monomeric MRF to form the active ternary complex. Since the E12 monomer-DNA complex shows negative cooperativity for the binding of a second E12 monomer, the formation of the heterodimer-DNA complex is preferred. Moreover, there is an enhancement of the overall affinity of both heterodimers compared to either the MyoD or Myogenin homodimers. Taken together, these analyses indicate that the heterodimeric species of an MRF and E12 bound to DNA is the most stable of the possible species that could be involved in musclespecific gene regulation and that this complex forms in a stepwise fashion. This model agrees well with the conclusions of others (24, 26), specifically, Wendt et al. (24), who proposed a DNA-mediated folding of MyoD and E47.

The important role of protein—protein interactions in the formation of the active, ternary complex is underscored by the results of the binding studies with variant binding sites. In each case, the affinity of binding was reduced significantly, but binding enhancement of the heterodimeric interaction, with respect to the homodimeric complex, is observed if the spacing between the half-sites is preserved. Thus, proper formation of the heterodimeric species is driven by the DNA sequence.

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