MyoD−E12 Heterodimers and MyoD−MyoD Homodimers Are Equally Stable[†]

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Received February 4, 1997; Revised Manuscript Received April 3, 1997[⊗]

ABSTRACT: Muscle development is controlled by the MyoD family of basic helix−loop−helix (bHLH) DNA-binding proteins. These proteins dimerize with ubiquitous products of the E2A gene (E12 and E47) and bind in a sequence-specific manner to enhancer regions of muscle-specific genes activating their expression. In this study, fluorescence anisotropy has been utilized to characterize the interactions of recombinant MyoD and E12 in solution in the absence of DNA. The Gibb's free energies of dissociation (ΔG) and the equilibrium dissociation constants (K_D) for the protein−protein interactions are reported. The ΔG for the MyoD homodimers in 100 mM KCl was 8.7 kcal/mol ($K_D = 340$ nM), and increasing the salt concentration resulted in destabilization of the dimer. From titrations of MyoD−dansyl with E12 at 100 mM KCl, a free energy of heterodimerization of 8.7 (+0.4/−2.4) kcal/mol was recovered using rigorous confidence limit testing. The titrations of E12-dansyl with MyoD yielded a free energy of 8.3 kcal/mol with tighter confidence limits, +0.5/−0.8 kcal/mol. Thus, in the absence of DNA, both MyoD homodimers and MyoD−E12 heterodimers are relatively weak complexes of approximately the same stability. E12 does not form stable homo-oligomeric complexes; remaining monomeric at concentrations as high as 20 μM. Based on these results and the apparent binding constants reported previously for DNA binding, DNA is likely to facilitate the dimerization of MyoD and E12. Furthermore, higher affinity interactions of MyoD−E12 heterodimers versus MyoD homodimers with DNA binding sites is not due to preferential heterodimerization.

The MyoD family of proteins (MyoD, myogenin, Myf-5, and MRF4) are dominant transcriptional regulators that control the expression of muscle-specific structural genes (Davis *et al.*, 1987; Edmondson & Olson, 1989; Braun *et al.*, 1989; Rhodes & Konieczny, 1989; Wright *et al.*, 1989). Overexpression of any one of these protein factors in non-muscle cell lines, such as NIH3T3 and CH310T1/2 fibroblasts, results in phenotypic conversion of those cells to muscle (Braun *et al.*, 1989; Yutzey *et al.*,1990). These myogenic transcription factors (referred to as the myogenic helix−loop−helix proteins, mHLH) are members of a larger family of proteins that are homologous over a 60 amino acid, basic helix−loop−helix (bHLH), domain [Murre *et al.*, 1989a; reviewed in Dias *et al.* (1994)]. The crystal structure of the bHLH domain of the MyoD homodimer−DNA complex has been solved revealing a number of specific residues involved in DNA binding and dimerization (Ma *et al.*, 1994). bHLH proteins interact with a DNA target termed an E-box, with the consensus CANNTG, which is present in the control regions of numerous muscle-specific genes. This sequence-specific interaction is mediated primarily through the basic domain of the bHLH motif, which is also involved in transcriptional activation.

mHLH proteins have been found to interact with other members of the bHLH family such as the ubiquitous products of the *E2A* gene (E12 and E47) through the highly conserved HLH motif (Tapscott *et al.*, 1988). The crystal structure of E47 bHLH dimer−DNA complex (which cannot activate myogenesis) has been solved and compared to MyoD−bHLH−DNA providing insight into subtle differences that may affect dimerization and site specificities of the bHLH proteins (Ellenberger *et al.*, 1994). EMSA (electrophoretic mobility shift assay) analysis of E12 demonstrated an inhibitory domain, encoded by an exon different than that in E47, which impairs homo-oligomerization (Sun & Baltimore, 1991a,b). Mutational analysis revealed that two glutamic acid residues and one arginine residue in helix 1 of the HLH domain were critical for the inhibitory domain function (Shirakata & Patterson, 1995). Heterodimers of the mHLH proteins with E12/E47 have been shown to bind E-boxes with higher affinity than the corresponding homodimers (Murre *et al.*, 1989b; Hu *et al.*, 1992). Muscle differentiation can be inhibited by *E2A* antisense expression vectors (Lassar *et al.*, 1991). It has also been shown that MyoD antibodies co-precipitate the *E2A* gene products along with MyoD from nuclear extracts of myofibers, confirming the observation that these proteins interact *in vivo* (Lassar *et al.*, 1991). Additionally, co-transfection experiments have demonstrated the ability of the mHLH proteins to interact with the ubiquitous products of the *E2A* gene *in vivo*. These heterologous interactions have been reported to enhance DNA binding (Sun & Baltimore, 1991a,b; Lassar *et al.*, 1991;

[†] This work was supported by grants from the American Heart Association-Arkansas Affiliate (B.K.H.), the American Cancer Society (B.K.H.), the U.S. Department of Energy EPSCoR program (S.J.M.), and the UAMS College of Medicine (S.J.M.).

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[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1997.

[1] Abbreviations: EMSA, electrophoretic mobility shift assay, MSV, murine sarcoma virus; bHLH, basic helix−loop−helix; mHLH, myogenic helix−loop−helix; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin.

S0006-2960(97)00262-6 CCC: $14.00

Shirakata & Patterson, 1995) and, therefore, transcriptional activity associated with these proteins. The protein Id ("inhibitor of differentiation"), which contains a HLH dimerization domain but lacks the basic domain, is thought to inhibit DNA binding of the mHLH proteins by dimerizing with the *E2A* proteins and sequestering them (Benezra *et al.*, 1990). Therefore, interactions of the mHLH and their dimerization partners [E12/E47 and MTwist, a recently reported inhibitor of the mHLH proteins (Spicer *et al.*, 1995)] are crucial for regulation of gene expression. Taken together these observations have led to the notion that mHLH proteins do not homodimerize as well as they heterodimerize (Sun & Baltimore, 1991a,b).

The pattern of expression throughout development and the *in vivo* biological roles of the mHLH proteins have been studied extensively [recently reviewed in Megeney and Rudnicki (1995) and Rudnicki and Jaenisch (1995)]. However, relatively few quantitative studies on the macromolecular interactions among the mHLH proteins and their binding partners, E12/E47, have been reported. In this study, we employed fluorescence anisotropy to analyze the interactions between highly purified recombinant MyoD and E12 in the absence of DNA. The application of fluorescence anisotropy to macromolecular interactions has been recently reviewed (Lundblad *et al.*, 1996; Heyduk *et al.*, 1996). This solution-based method allows the study of macromolecular interactions directly under true equilibrium conditions and is particularly suited to the characterization of relatively weak interactions. We report that the predominant homo-oligomeric form of MyoD below 30 μM is a dimer. Also, without a heterodimerization partner, the monomeric form of E12 is the predominant species in solution even at high concentrations. Finally, in the absence of DNA, MyoD homodimers exhibit approximately the same stability as heterodimers of MyoD−E12. Thus, the observed higher affinity DNA binding by MyoD−E12 heterodimers over MyoD homodimers is not a consequence of preferential heterodimerization.

EXPERIMENTAL PROCEDURES

MyoD and E12 Proteins. Mouse MyoD and human E12 proteins were overexpressed in *Escherichia coli* strain BL21-(DE3)pLysS from T7 expression plasmids. Plasmids pT5-MyoD and pT5-E12 were generous gifts of Charles P. Emerson, Jr. (University of Pennsylvania School of Medicine). The expression and purification of MyoD and E12 have been described elsewhere (Maleki & Hurlburt, 1997). The proteins were judged to be 95% pure following two-dimensional gel electrophoresis and Coomassie Brilliant Blue staining. Small aliquots of purified protein were stored at −80 °C and freshly thawed for each experiment. The concentrations of MyoD and E12 were determined by absorbance at 280 nm using extinction coefficients of 17 750 and 17 900 M^{-1}, respectively, which were calculated using the method described by Gill and von Hippel (1989).

DNS Labeling. Aliquots of proteins for anisotropy measurements were thawed on ice and desalted into 250 mM $NaPO_4$ buffer, pH 8, using 10DG pre-calibrated columns (BioRad). The desalted proteins were labeled with dansyl chloride (DNS) according to methods described by Fernando and Royer (1992). At pH 8, the NH_2-terminal residue is the predominant point of probe linkage. Following DNS labeling, the proteins were applied to a Sephadex G-25 superfine column that was pre-equilibrated with SJM buffer (10 mM Hepes/KOH, pH 7.9, 1 mM EDTA, 5 mM dithiothreitol, 5% glycerol). The elution profile showed clear separation between free label and excluded protein, which was collected in three approximately 1-mL fractions. The most concentrated fractions were used directly in fluorescence anisotropy assays after the addition of desired amounts of KCl. All experiments were performed in the presence of 100 mM KCl unless indicated otherwise. The stoichiometries of labeling for the MyoD-DNS and E12-DNS preparations were determined as described (Fernando & Royer, 1992) and ranged from 1.0 to 1.2 DNS/protein. The activity of the DNS-labeled proteins in DNA binding was compared to that of the wild type and found to be essentially unchanged (data not shown).

Protein Cross-Linking. Protein aliquots were desalted into 100 mM $NaPO_4$, pH 8.0, using 10DG columns as above. The protein cross-linking reagents dithiobis(succinimidyl propionate) (DSP), disuccinimidyl suberate (DSS), and bis-(sulfosuccinimidyl)suberate (BS^3) (Pierce Chemical Co.) were used. BS^3 was dissolved in water, and DSP and DSS were dissolved in DMSO as 10 mM stock solutions. A 1-μL sample of cross-linker stock solution was added to the 20 μL reaction. Cross-linking reactions were carried out at a final protein concentration of 10 μM in the absence and presence of a double-stranded 24 base-pair DNA (10 μM) containing the muscle creatine kinase (MCK) enhancer right E-box described in Maleki and Hurlburt (1997). The reactions were quenched after 30 s by addition of SDS−sample buffer (lacking DTT for the DSP reactions), heated to 100 °C for 5 min, and subjected to SDS−polyacrylamide gel electrophoresis (SDS−PAGE). In developing this assay, titrations of the cross-linking reagent, titrations of MyoD, and time courses with constant amounts of cross-linking reagent were performed to establish the most appropriate conditions for the experiment. Extended times of cross-linking or increased concentrations of reagent led to very large aggregates of protein that were essentially unable to enter the SDS gel. Specific oligomeric species greater than a dimer were not observed under any of the conditions tested. Therefore, we concluded that the specific oligomer was a dimer and that further treatment with the cross-linking reagents led to nonspecific complexes. Limited cross-linking was performed to minimize the formation of the nonspecific complexes.

Immunodetection. Cross-linked proteins were resolved by SDS−PAGE and electrophoretically transferred to nitrocellulose. MyoD was detected by Western blot analysis using monoclonal antibody 5.8A (Dias *et al.*, 1992), generously provided by Peter Dias and Peter Houghton (St. Jude Children's Research Hospital). The nitrocellulose filters were blocked with 5% nonfat dry milk, 1% BSA in TBST (Tris-buffered saline, 0.05% TWEEN-20) for 30 min at room temperature. TBST washed filters were incubated with primary antibody (1:1000 dilution) in TBST for 1 h followed by incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5000 dilution) for 30 min in TBST−5% milk. The secondary antibody was detected by chemiluminescence.

Fluorescence Anisotropy Assays. This technique has been described in detail by Fernando and Royer (1992). All fluorescence assays were carried out at 21 °C in SJM buffer

containing 100 mM KCl unless otherwise indicated. Anisotropy measurements were performed using an ISS KOALA fluorescence polarization spectrometer in L format (Champaign, IL). An ILC Technologies 300-W xenon arc lamp (Sunnyvale, CA) was used to excite the dansyl label at 350 nm with 8 mm bandwidth and using a Y460 cuton filter (Hoya Optics, Freemont, CA) in emission. The background fluorescence with all added components, including unlabeled protein (MyoD and/or E12) at the same concentration as in the experiments was subtracted for every sample during analysis of both homo- and hetero-oligomer interactions. The decrease in intensity upon dilution of labeled protein was normalized by dividing the intensity by the protein concentration for all assays except when a constant amount of labeled protein was titrated. The anisotropy values reported were measured only over the invariant range of normalized intensity. Anisotropy determinations were performed at least three times for each experiment.

Fluorescence Anisotropy of MyoD and E12. Although a complete description of all of the linked equilibria of the myogenic regulatory factors, including those with DNA, is a goal of our research, this report concentrates on the interactions between MyoD and E12 in the absence of a DNA ligand using the steady-state anisotropy of a covalently bound fluorophor, DNS. The steady-state fluorescence anisotropy depends on the rate of rotational diffusion of the fluorophor. Under conditions where the local environment of the fluorophor does not change, 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 [see Fernando and Royer (1992), Lundblad *et al.* (1996), and Heyduk *et al.* (1996) for complete descriptions of the application of fluorescence anisotropy to macromolecular interactions]. Briefly, plane polarized light is used to excite a fluorophor. Anisotropy (A), which is directly related to the size of the complex, is calculated from the intensity of the fluorescent emissions in the planes parallel ($I_{||}$) and perpendicular ($I_{\perp}$) to the excitation plane using the following equation:

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

The anisotropy is related to the rotational correlation time (τ_c) of the fluorophor through the Perrin equation, where τ is the fluorescence lifetime and A_o is the limiting anisotropy of the fluorophor:

$$\frac{A_o}{A} - 1 = \frac{\tau}{\tau_c}$$

In the case of a fluorophor covalently bound to a macromolecule, the anisotropy is indicative of both the local motions of the fluorophor and the global tumbling of the macromolecule. However, since the local motions are not concentration dependent, any change in anisotropy as a function of protein concentration necessarily arises from an oligomerization equilibrium.

Homo-Oligomer Interactions. The fluorescence anisotropy of serial dilutions (0.5 and 0.8 dilutions) of a homogeneous population of concentrated, DNS-labeled MyoD or E12 was measured to analyze homo-oligomer formation. In the case of MyoD, anisotropy measurements were done in SJM buffer containing various salt concentrations ranging from 0 to 300 mM KCl.

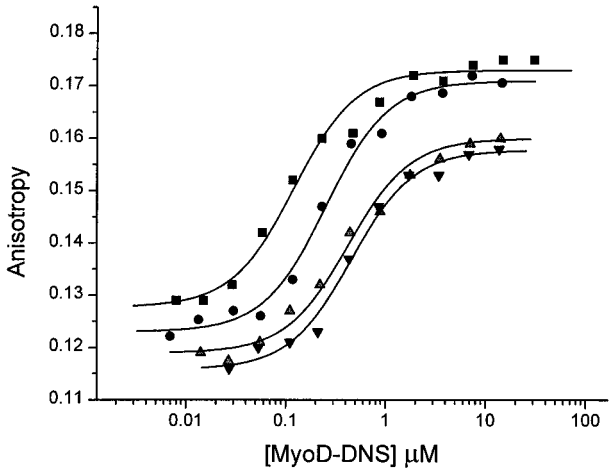

FIGURE 1: Fluorescence anisotropy of MyoD−MyoD interactions. The fluorescence anisotropy of dansyl-labeled MyoD (MyoD-DNS) at various concentrations is plotted versus the concentration of MyoD-DNS. The samples were in SJM buffer plus various concentrations of KCl as indicated: (■) 0 mM KCl, (●) 100 mM KCl, (▲) 200 mM KCl, (▼) 300 mM KCl. All assays were performed at 21 °C.

Hetero-Oligomer Interactions. A constant amount of DNS-labeled MyoD (40 nM) or E12 (40 nM) in SJM buffer was incubated with various concentrations of unlabeled E12 and MyoD, respectively. The anisotropy of fluorescence of every sample was measured and plotted versus the concentration of unlabeled protein.

Analysis of Anisotropy Data. Binding isotherms, generated from the values of fluorescence anisotropy as a function of protein concentration, were fit to a model of monomer $\rightleftarrows$ dimer equilibrium using a numerically based algorithm (BIOEQS, Royer *et al.*, 1991; Royer & Beechem, 1992; Royer, 1993). BIOEQS solves directly for the species populations based on the values of the Gibb's free energies of dissociation (ΔG) for the complexes and thus requires no closed form analytical expression for the binding isotherm. The model used for the homodimerization included the following three free parameters; the ΔG of dissociation of the dimer of MyoD to free monomers and the plateau values of the anisotropy of the monomeric and dimeric MyoD-DNS species. The model for the heterodimerization studies was more complex. The floating parameters included ΔG for the heterodimer of MyoD−E12 and the plateau values of the anisotropies of the relevant monomeric and dimeric labeled species ΔG for MyoD homodimer was fixed. The free energies were optimized using a non-linear, least squares algorithm. Rigorous confidence limit testing (Royer *et al.*, 1991; Beechem, 1992) was performed on the recovered free energy values over a range of at least 4 kcal/mol in 0.1 kcal/mol increments, allowing the plateaus to be adjusted by the non-linear optimization routine. The equilibrium dissociation constants were calculated from the ΔG values using $\Delta G = -RT \ln K$.

RESULTS

Analysis of MyoD Homo-oligomerization. To analyze the interactions between MyoD monomers, the fluorescence anisotropy of various concentrations of purified MyoD, labeled with dansyl chloride (MyoD-DNS), were determined as a function of KCl concentration between 0 and 300 mM (Figure 1). The plateau seen above 1 μM MyoD indicated

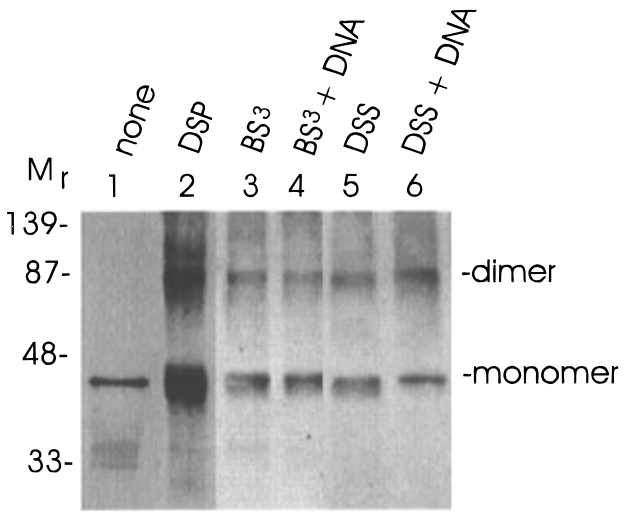

FIGURE 2: Limited cross-linking of MyoD. MyoD at 10 μM was briefly treated with three cross-linking reagents: BS^3, DSS, and DSP to establish the stoichiometry of the complex at the upper plateau. The cross-linking reagents and the presence of the MCK enhancer oligonucleotide (DNA) are indicated The reactions were quenched with sample buffer, and the proteins were resolved by SDS−PAGE, transferred to nitrocellulose, and detected with a monoclonal antibody to MyoD. Chemiluminescence was used for detection.

the presence of a homogeneous species. At concentrations of MyoD below 1 μM, a decrease in anisotropy was observed which indicates that the homogeneous species between 1 and 10 μM was dissociating into smaller component molecules, presumably monomers. Also, increasing the salt concentration from 0 to 300 mM resulted in destabilization of the complex, consistent with the notion that electrostatic interactions contribute to MyoD homo-oligomerization (Ma *et al.*, 1994; Shirakata & Patterson, 1995). It should be noted that the anisotropy values reported were for samples in which the intensity of the probe did not change; e.g., the intensity, normalized for the dilution factor, was constant, thus the local environment of the probe was unaltered. The decrease in anisotropy plateaus with increasing salt concentration is likely due to small changes in the local motion of the fluorophor.

The stoichiometry of the predominant homo-oligomeric MyoD complex present at the upper plateau (above 1 μM) was established by limited chemical cross-linking with three different agents DSP, BS^3, and DSS (see Experimental Procedures for details) in the presence and absence of a specific DNA ligand (Figure 2). Cross-linked MyoD was resolved by SDS−PAGE, transferred to nitrocellulose, and probed with anti-MyoD monoclonal antibody. Immunodetection was utilized because MyoD negatively stains with silver. As shown in Figure 2, limited cross-linking results in the formation of an electrophoretically stable complex with an apparent molecular mass of approximately 90 kDa, appropriate for a MyoD homodimer in SDS−PAGE. Cross-linking for longer periods of time or at higher concentrations of cross-linking reagents resulted in the formation of protein aggregates that either did not enter or did not migrate far in the SDS gel (data not shown). There were no conditions tested that resulted in the formation of discreet, resolvable oligomers with an apparent molecular mass exceeding 90 kDa. The X-ray crystallographic structure of MyoD bHLH domain bound to DNA target site as well as biochemical analyses indicate that MyoD binds to DNA as a dimer (Ma *et al.*, 1994; Shirakata & Patterson, 1995). Therefore, we

Table 1: ΔG and K_D Values for MyoD and E12 Interactions at 21 °C

protein	[KCl] (mM)	ΔG (kcal/mol) of dissociation	K_D (M)
MyoD*/MyoD*	0	9.3 (±0.5)	1.2×10^{-7}
MyoD*/MyoD*	100	8.7 (±0.5)	3.4×10^{-7}
MyoD*/MyoD*	200	8.6 (±0.5)	4.0×10^{-7}
MyoD*/MyoD*	300	8.4 (±0.5)	5.7×10^{-7}
E12*/E12*	100	ND[b]	
MyoD*/E12	100	8.7 (+0.4/−2.4)	3.4×10^{-7}
E12*/MyoD	100	8.3 (+0.5/−0.7)	6.7×10^{-7}

[a] An asterisk (*) indicates the protein labeled with dansyl. [b] Could not be determined.

cross-linked MyoD in the presence of a synthetic DNA fragment corresponding to the right E-box of the muscle creatine kinase (MCK) enhancer (Maleki & Hurlburt, 1997). In Figure 2, the oligomers of MyoD in lanes 4 and 6 (with DNA) are not discernibly different than those in lanes 3 and 5 (without DNA), leading to the conclusion that the predominant oligomer formed at 10 μM MyoD in the presence or absence of DNA is a dimer.

Based on the cross-linking results, the data from Figure 1 were fit to a model of monomer $\rightleftarrows$ dimer equilibrium using a numerically based algorithm, BIOEQS (see Experimental Procedures). The Gibb's free energies of dissociation (ΔG) for the dimer were recovered from the non-linear, least squares analysis of the data, and the errors reported are the results of rigorous confidence limit testing, as described in Experimental Procedures. The equilibrium dissociation constants (K_D) were calculated from the recovered free energy values (Table 1). We found that the dissociation constant for MyoD dimerization in solution in 100 mM KCl is approximately 400 nM, which is considerably higher than apparent K_D values reported for MyoD homodimers binding DNAs with single E-boxes [Maleki, S. J., Royer, C. A., and Hurlburt, B. K. (in preparation); Sun & Baltimore, 1991a,b].

E12 Homo-Oligomerization. Based primarily on EMSA data, E12 has been reported to not form stable homo-oligomers (Sun & Baltimore, 1991a,b; Shirakata & Patterson, 1995). Here, homo-oligomerization of E12 was examined directly in the absence of DNA. The fluorescence anisotropy of various concentrations of purified, dansyl-labeled E12 (E12-DNS) were determined in SJM buffer with 100 mM KCl (Figure 3). A very small change in anisotropy (<0.02) was observed, indicating that there was no significant difference in the oligomeric state of E12 between 40 nM and 20 μM. This result provides direct evidence that E12 does not form stable oligomers even at high concentrations, confirming previously reported conclusions (Sun & Baltimore, 1991a,b; Shirakata & Patterson, 1995). Limited cross-linking of a 10 μM solution of E12, as described for MyoD above, revealed no oligomers (data not shown).

MyoD and E12 Hetero-oligomerization. E12 has been shown to be a natural dimerization partner of MyoD *in vivo* (Lassar *et al.*, 1991). The heterodimers of these proteins have been shown to bind E-boxes with higher affinity than homodimers of MyoD (Sun & Baltimore, 1991a,b). Accordingly, it has been proposed that MyoD−E12 heterodimers are more stable than MyoD homodimers. To examine hetero-oligomerization of MyoD and E12 directly, a limiting amount of MyoD-DNS (40 nM) was titrated with unlabeled E12 and the fluorescence anisotropy measured

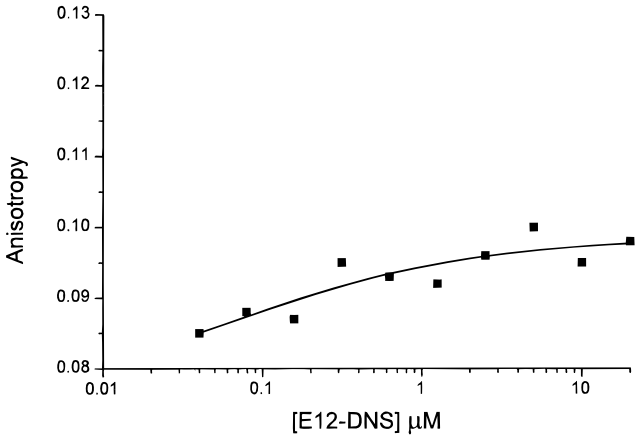

FIGURE 3: Fluorescence anisotropy of E12−E12 interactions. The fluorescence anisotropy of dansyl-labeled E12 (E12-DNS) at various concentrations is plotted versus the concentration of E12-DNS. The samples were in SJM buffer plus 100 mM KCl. All assays were performed at 21 °C.

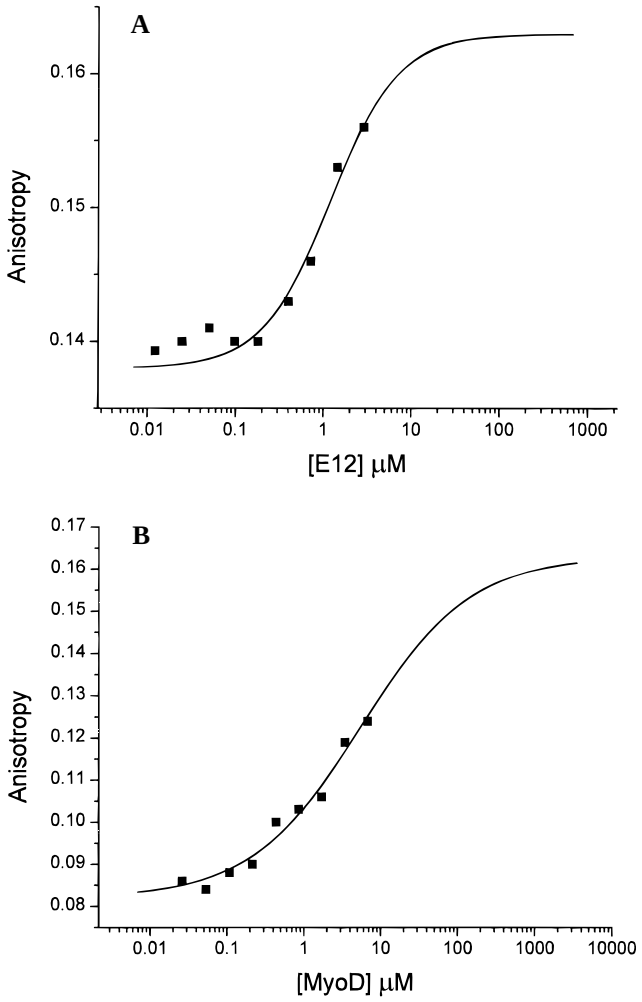

FIGURE 4: Fluorescence anisotropy of MyoD−E12 interactions. Dansyl-labeled protein is maintained at a constant concentration (40 nM) and equilibrated with various concentrations of either MyoD or E12. The samples were in SJM buffer plus 100 mM KCl. (A) Dansyl-labeled MyoD (MyoD-DNS) is titrated with unlabeled E12. The fluorescence anisotropy is plotted versus the concentration of E12. (B) Dansyl-labeled E12 (E12-DNS) is titrated with MyoD. The fluorescence anisotropy is plotted versus the concentration of MyoD. All assays were performed at 21 °C.

(Figure 4A). The reciprocal experiment was also performed in which a limiting amount of E12-DNS (40 nM) was titrated with unlabeled MyoD and the anisotropy measured (Figure 4B). Limited cross-linking of MyoD−E12 at 10 μM indicated that dimers were the predominant hetero-oligomer (data not shown). The data were fit using BIOEQS (as described in Experimental Procedures) using a model that included MyoD homodimers, MyoD−E12 heterodimers, and the free MyoD and E12 monomers. The apparent ΔG and K_D values from the fits are presented in Table 1. Saturation of the labeled protein did not occur, even at the highest concentrations of soluble titrant protein available. Rigorous confidence limit testing revealed that the free energy of heterodimer dissociation recovered from the fit of the MyoD-DNS titrations with E12 of 8.7 kcal/mol could be taken with asymmetric confidence limits of +0.4 kcal/mol and −2.4 kcal/mol because of the limited number of data points in the transition region. The upper and lower plateau values recovered were 0.163 and 0.13, respectively. It should be noted that the slightly higher plateau value for MyoD-DNS in Figure 3 versus Figure 1 is because the concentration of MyoD-DNS in Figure 3 (40 nM) is not at the lower plateau in Figure 1. This concentration was chosen in order to ensure having a strong fluorescent signal from the DNS probe throughout the titration with E12. At high concentrations of either titrant protein, light scattering increased the observed fluorescent intensity, which was corrected for using unlabeled protein samples as blanks.

The analysis of the titration of E12-DNS with MyoD yielded a ΔG of heterodimerization of 8.3 kcal/mol also with asymmetric but tighter confidence limits of +0.5 and −0.8 kcal/mol. The larger confidence limits on the lower limit of the absolute value of the free energy result from the lack of a high concentration plateau in the data and the fact our confidence limits testing procedure allows for an error to be estimated taking into account the correlation between the free energy and the plateau values. The upper and lower plateau values recovered were 0.169 and 0.082, respectively. Titration of E12-DNS with unlabeled MyoD (Figure 4B) occurred over a larger range of concentration than the reciprocal experiment due to competition with MyoD−MyoD homodimerization. From the values reported in Table 1, we conclude that the free energy of MyoD−E12 heterodimer formation under our conditions is approximately equal to that of MyoD−MyoD homodimer formation, but much stronger that E12 oligomerization.

DISCUSSION

MyoD is representative of a family of transcriptional activators that utilizes a conserved basic helix−loop−helix motif (bHLH) for dimerization and DNA binding. The expression pattern of the muscle-specific bHLH proteins (MyoD, myogenin, Myf-5, and MRF4) throughout development and identification of their dimerization partners (e.g., E12, E47, and Id) as well as their DNA binding sites are well described. However, basic questions about the thermodynamic linkages of the interactions between these transcription factors, their dimerization partners, and their association with DNA remain unanswered. Here we report the Gibb's free energies of dissociation (ΔG) and equilibrium dissociation constants (K_D) at 21 °C for the interactions of MyoD and one of its *in vivo* partners, E12, as determined by fluorescence anisotropy under equilibrium conditions. We have shown that, in the absence of DNA at 100 mM KCl, MyoD homodimers exhibit approximately the same stability

as MyoD−E12 heterodimers. Homodimers of MyoD are destablized by increasing salt, confirming an electrostatic contribution to subunit interactions suggested by crystallographic studies (Ma *et al.*, 1994). Also, E12 does not form stable homodimers, remaining monomeric at concentrations up to 20 μM. Chemical cross-linking indicated that dimers of MyoD predominate at 10 μM. This result is in agreement with the cross-linking data reported by Lin and Konieczny (1992), who observed homodimers of MyoD and heterodimers of MyoD−E12. However, it has also been reported that the monomeric MyoD is in reversible equilibrium with micelles of more than 100 monomers with a small percentage of the MyoD observed to have an apparent mass between that of the monomer and a tetramer (Laue *et al.*, 1995). Under our conditions, MyoD irreversibly forms aggregates at concentrations above 50 μM (Maleki & Hurlburt, 1997), not reversible micelles. Furthermore, micelles would be relatively large, tumble relatively slowly in solution, and would likely have a higher anisotropy than we observed.

Our previous (Czernik *et al.*, 1996) and ongoing studies as well as those reported by Sun and Baltimore (1991a,b) indicate that the apparent K_D values for MyoD homodimer−DNA interactions and MyoD−E12 heterodimer interactions are very low in comparison to the values reported here for dimer formation in the absence of DNA. Thus, MyoD and E12 are likely to rely on DNA binding to facilitate dimerization. We have observed positive cooperativity in binding of MyoD−E12 to individual E-boxes of the murine sarcoma virus enhancer (Czernik *et al.*, 1996). DNA-facilitated protein oligomerization is well documented for prokaryotic (Ptashne, 1986; Wong *et al.*, 1992) and eukaryotic (Lamb & McKnight, 1991) DNA-binding proteins. Detailed studies on the *E. coli* Rep helicase (Wong *et al.*, 1992) and the glucocorticoid receptor (Leftsin *et al.*, 1994) revealed allosteric contributions to protein oligomerization on the DNA binding site. Moreover, Spolar and Record (1994) have reported that DNA binding induces folding of flexible or relatively unfolded protein subdomains for several well-characterized proteins. Interestingly, the bHLH domain of MyoD has been reported to increase in α-helical content upon DNA binding (Anthony-Cahill *et al.*, 1992).

Whereas the four mHLH proteins are highly homologous and have equivalent activities in most transfection assays, primarily developmental expression profiles, transgene, and null mutant studies have shown that these proteins have distinct roles in myogenesis and muscle-specific gene regulation. The homodimerization and heterodimerization preferences of these transcription factors with ubiquitous bHLH proteins such as E12, E47, Id, and MTwist may be significant determinants of functional distinctions among them. However, the results presented here indicate that the increased affinity of MyoD−E12 heterodimers for E-box containing enhancer DNAs over MyoD homodimers is not due to enhanced stability of the heterodimer. Post-translational modifications may play a crucial role in the dimerization preferences of the bHLH proteins. These aspects of DNA binding specificity for the mHLH proteins are currently under investigation.

ACKNOWLEDGMENT

We are indebted to Paul Gollnick, Randy Haun, and Kevin Raney for critiques on the manuscript. We thank Ross Reedstrom and John Hill of the Royer lab for technical assistance and Peter Dias and Peter Houghton for providing the anti-MyoD monoclonal antibody.

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BI970262M