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KINETIC ANALYSIS OF B/HLH/Z TRANSCRIPTION FACTORS MYC, MAX AND MAD INTERACTION WITH COGNATE DNA[†]

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Abstract

Myc, Mad and Max proteins belong to the basic helix-loop-helix leucine zipper family of transcription factors. They bind to a specific hexanucleotide element of DNA, the E-box (CACGTG). In order to be biologically active, Myc and Mad require dimerization with Max. For the route of complex assembly of these dimers, there are two proposed pathways. Monomer pathway: Two monomers bind DNA sequentially and assemble their dimerization interface while bound to DNA. Dimer pathway: Two monomers form a dimer first prior to association with DNA. The monomer pathway is kinetically favored. In this report, stopped flow polarization was utilized to determine the rates and temperature dependence of all of the individual steps for both assembly pathways. Myc·Max dimerization had ~5-fold and ~2-fold higher rate constant than Max·Max and Mad·Max, respectively. The protein dimerization rates as well as the dimer-DNA rates were found to be concentration independent suggesting conformational changes were rate limiting. The Arrhenius activation energies for the dimerization of Myc, Mad and Max interaction with Max were 20.4 ± 0.8 , 29 ± 0.6 and 40 ± 0.2 kJ/mol, respectively. Further, rate constants for Max·Max homodimer DNA binding are significantly higher than for Myc·Max and Mad·Max heterodimers binding to DNA. Monomer-DNA binding showed a faster rate than dimer-DNA binding. These studies show the rate-limiting step for the dimer pathway is the formation of protein dimers and this reaction is slower than formation of protein dimers on the DNA interface, kinetically favoring the monomer pathway.

Myc, Max and Mad are members of the basic helix-loop-helix leucine zipper family of transcription factors. Myc was first discovered as the protooncogene of avian retroviruses inducing lymphoid tumors (1). It is believed to regulate 15% of all genes (2). Deregulation of Myc has been implicated in the development of many human cancers, including Burkitt lymphoma, neuroblastomas, small cell lung cancers, breast cancers, oesophageal, adenocarcinoma and medulloblastomas (3,4). c-Myc is over expressed in neoplasia by a number of different mechanisms, including gene amplification, translocation, retroviral insertion, and activation of pathways upstream of c-Myc expression (5). The Myc family proteins, c-Myc, n-Myc and l-Myc, have been implicated almost exclusively in cell proliferation, differentiation and neoplasia (6–10). However, recent advances have shown that c-Myc is involved in a wide catalog of cellular activity, which includes recruitment to the transcriptosome of a variety of transcriptional effectors including TRRAP (transformation/transcription) domain associated protein, Miz1 (transcriptional repressor), and the E2 ubiquitin ligase Skp2; and activates the cad gene that encodes the trifunctional enzyme carbamoyl-phosphate synthase/aspartate

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transcarbamoylase/dehydroorotase, which is required for the first three rate-limiting steps of pyrimidine biosynthesis (11–13).

Since neither dimerization nor DNA specific binding could be readily demonstrated for Myc protein, a search for Myc interacting proteins led to the identification of Max protein. The Mycobligate factor X, Max, is a b/HLH/Z¹ family protein similar to Myc, but lacks the transactivation domain. Max can also form homodimers. Studies showed that it may act as a transcription repressor in a homodimer form (14). All known oncogene functions of Myc require dimerization with Max. The Myc·Max transcription activator is involved in the transcriptional regulation of target genes associated with cellular growth, proliferation, metabolism and differentiation (7,15). The fact that Max is expressed in the absence of Myc lead to searches for other Max-interacting partners. Mad family proteins were all identified in expression cloning screens by their ability to bind specifically to Max (16–18). Similarly to Myc, Mad homodimerizes poorly but interacts with Max, forming a sequence-specific DNA binding complex similar to Myc·Max heterodimer. Overexpression of Mad in tissue culture and mice interferes with cell proliferation and inhibits transformation (15). Mad and Myc compete for binding to Max.

The x-ray crystal structures of the b/HLH/Z domains of Myc·Max and Mad·Max revealed that both heterodimers bind to their common DNA target, the enhancer box (E box) hexanucleotide (5'-CACGTG-3') (8,19). E-boxes are located in the proximal region of class II nuclear gene promoters, between 50 and 200 base pairs upstream of the transcription sites. Binding of these structurally similar transcription factor dimers to the E-Box determines whether a cell will divide and proliferate (Myc·Max) or differentiate and become quiescent (Mad·Max). Many transcriptional factors form dimeric complexes with DNA (20,21). In the absence of DNA, they may be found either as monomers (22) or dimers (23). Homodimer/heterodimer of Max·Max, Myc·Max, and Mad·Max transcription factors recognized DNA E-box (24). The discrimination between cognate and nonspecific Max b/HLH/Z/DNA complex have been reported using EMSA, CD, NMR techniques (25,26) and crystal structures using X-ray crystallography (27). Mass spectrometry/peptide analysis has also been employed to characterize Max binding to specific and non-specific DNA (28).

Given the ever expanding catalog of c-Myc functions as a transcription factor, we in this laboratory ascertained that little quantitative were available regarding the thermodynamic cycle of how the Myc/Max/Mad network behaves in relation to their protein-protein interactions and to the E box. In this study, as in our previous work, the b/HLH/Z domains of Myc and Max and full length Mad1 were studied (see materials). In our first study, Hu et al. (2005) using fluorescence anisotropy showed that specific binding between MLP DNA and Max was about 10-fold higher affinity than LCR DNA and about 100 fold higher than for non-specific DNA. USF had a similar binding affinity as Max to MLP DNA (29,30) but Max bound more tightly to LCR and non-specific DNA. A series of E-Box alterations showed for Max binding that most of the energy involved could be attributed to the two halves of the E-box showing virtually no cooperativity. Measurement of binding for the entire thermodynamic cycle of monomer-dimer-DNA interactions showed that monomer-Max-DNA complex showed a significantly reduced affinity for the second monomer of Max. Our work showed that Myc·Max formed the most stable heterodimer. Further, Banerjee et al. (2006) also from this laboratory showed that formation of a dimer complex might be affected by a general environment with respect to charge. Thus by fluorescence anisotropy it was shown that poly-L-lysine had an insignificant

¹Abbreviations: b/HLH/Z, basic helix-loop-helix leucine zipper; DNA E-Box, six base pair double-stranded DNA sequence CACGTG known as enhancer box; MLP, major late promoter; LCR, human β -globin locus control region; FITC, fluorescein isothiocyanate; IPTG, isopropyl β -D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PBS, phosphate buffer saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate, DMSO, dimethyl sulfoxide.

effects, poly-L-glutamic acid stabilized both homodimers and heterodimers. These results showed that dimerization of all three monomers in a negatively charged milieu were driven by both negative enthalpy and entropic contributions. van der Waals and H-bonding interactions are most likely at play in the formation of these complexes.

Kinetic analysis has shown that assembly of the dimeric transcription factors (bZIP and bHLHZip) and specific DNA sites follows a pathway in which two protein monomers bind DNA sequentially and form their dimerization interface while bound to DNA (monomer pathway) (31). The individual steps of this process provide kinetic control which will lead to different intermediates that can interact at different rates with other proteins and non-specific DNA and ultimately allow for discrimination between specific and non-specific DNA. Therefore, it is important to determine the rate limiting steps and kinetics of the individual steps in order to understand regulation of transcription by cellular and viral proteins and other pharmacological agents.

In this work, stopped-flow anisotropy techniques were utilized to study the dimerization kinetics of Max, Myc and Mad in the absence and presence of DNA E-box. The rate constants for Myc·Max, Mad·Max and Max·Max dimerization and the temperature dependence of these reactions were measured and compared. Our kinetic data showed that Myc·Max dimerization is faster than Max·Max or Mad·Max. The Myc, Max and Mad dimerization rates were found to be concentration independent suggesting conformational changes are rate limiting. In order to understand the assembly of transcription complex, we analyzed whether Myc or Mad competes to form a heterodimer with Max at the DNA E-box. Further, we measured and compared the dimer-DNA and monomer-DNA binding rate of the individual steps in the assembly pathway (monomer/dimer pathway) and determined the activation energies of the reactions.

EXPERIMENTAL PROCEDURES

Materials

GST-bind resin and HiTrap SP column were obtained from Amersham Pharmacia Biotech, Inc. His-bind Ni-resin was purchased from Novagen an affiliate of Merck Co. Ltd. Sephadex G-25 was purchased from Pharmacia Fine Chemicals Inc. Piscataway, N.J. Fluorescein isothiocyanate (FITC) was obtained from Molecular Probes, Inc. Oligonucleotides used in these studies were synthesized on a 200 nmol scale by Gene Link (Hawthorne, NY). Fluorescein was attached at the 5' end of the 16-mer oligonucleotides during the synthesis.

Expression and Purification of Proteins

Expression vectors Max/pET3a and c-Myc/pGEX2T were kindly provided by SK Burley, SGX Pharmaceuticals, Inc., San Diego, California. The cloning vector pET30a containing the full-length Mad cDNA was purchased from Open Biosystems, Huntsville, AL. All three vectors were transformed into *Escherichia coli* BL21 (DE3) pLysS cells that express T7 RNA polymerase from the IPTG-inducible lacUV5 promoter. Truncated proteins Max and c-Myc consisting of amino acids 22–113 and 347–439, respectively and the full-length Mad1 contained the functional b/HLH/Z domains (16,27,32). Cells containing pET Max or pGEX Myc were selected by colony selection and inoculated into 1L of LB media with 100 µg/ml ampicillin and grown at 37°C until the OD₆₀₀ reached 0.5–1. The culture was induced with 1 mM isopropyl β-D-thiogalactopyranose (IPTG) and the culture growth was allowed to continue for 6h at 30°C. Cells were harvested by centrifugation for 20 min, 6000 rpm and resuspended in lysis buffer (20 mM HEPES buffer, pH 7.6, 0.5 mM PMSF, 10% glycerol, protease inhibitor cocktail and lysozyme) and sonicated for 2 min with a 30 s interval. The lysed cells were centrifuged at 18000 rpm for 15 min to separate soluble proteins from inclusion bodies (Myc

and Mad1). The supernatant was used for the purification of the Max protein using ion exchange chromatography.

The inclusion bodies for Mad1 were isolated from cell lysates, washed twice with 10 ml of 20 mM HEPES buffer (pH 7.6) containing 1% Triton X-100. After centrifugation at 18000 rpm for 30 min, the inclusion bodies were dissolved in 2 ml of 50 mM HEPES buffer (pH 7.6), 6 M guanidine hydrochloride and 25 mM DTT and incubated for 1h at 4°C. To pellet the insoluble material, the sample was centrifuged at 18000 rpm for 10 min and the supernatant was diluted into 20 ml of 50 mM HEPES (pH 7.6).

The inclusion bodies for c-Myc were washed as described above for Mad1. The inclusion bodies were solubilized with 10 ml of phosphate buffer saline (PBS), pH 7.4 containing 1% Triton X-100 and incubated overnight with gentle stirring. After removal of the insoluble material by centrifugation, the soluble protein was diluted into 20 ml of cold 20 mM PBS, pH 7.4.

HiTrap SP-ion exchange chromatography was used for the purification of Max. c-Myc was purified by GST affinity chromatography using 20 mM PBS, pH 7.4 as the wash buffer and 200 mM Tris-HCl, pH 8.5, containing 50 mM glutathione as the elution buffer. GST was enzymatically removed from Myc. The purification of Mad1 was performed on a Ni²⁺-His trap affinity column. The column was prepared according to the manufacturer's instructions and washed with 10 volumes of binding buffer (20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl and 5 mM imidazole). The supernatant containing the Mad protein was loaded on the affinity column. The column was washed with additional 10 volumes of wash buffer (20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl and 60 mM imidazole), and the bound protein was eluted with the elution buffer (20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl and 1 M imidazole). The protein fractions were collected and dialyzed against HEPES buffer pH 7.6. The purity of all three proteins was determined by 10% SDS-polyacrylamide gel electrophoresis. After the proteins were concentrated with Centricon YM filters (Amicon Co.). The concentrations of protein were determined by a Bradford assay with bovine serum albumin as standard (33) using a Bio-Rad protein assay reagent (Bio-Rad Laboratory, CA). Protein purity was estimated to be >98% pure by examination of serial dilutions with SDS-polyacrylamide gel electrophoresis (34) and Coomassie Blue staining. Proteins were stored in small aliquots to avoid repeated thawing at frozen at -70 °C in storage buffer (10% glycerol, 1 mM PMSF, 100 mM KCl, 5 mM Hepes-KOH, pH 7.5, 10 mM DTT). No difference in activity (EMSA) was detected between freshly prepared and frozen samples. Electrophoretic mobility shift assays (EMSA) with E box containing oligonucleotides demonstrated high affinity, specific DNA binding activity for Myc-Max and Mad-Max heterodimers (data not shown and (19))

Fluorescent Labeling of Max

Fluorescein isothiocyanate (FITC) reacts only with uncharged primary amines of proteins to form dye-protein conjugates. Therefore, the N-terminus of Max was labeled with FITC as follows. The concentration of purified Max was adjusted to 1mg/ml in 50 mM HEPES buffer, pH 7.6. In the labeling procedure, 100 µl of 1.5mg/ml reactive dye stock solution (dissolved in anhydrous Dimethyl sulfoxide, DMSO) is added to 1ml of protein solution. The FITC-Max mixture was incubated overnight at 4°C with gentle agitation, protected from light. FITC-labeled Max was purified on a Sephadex G-25 column equilibrated with 50 mM HEPES buffer, pH 7.6. The FITC conjugated protein fractions were collected in the void volume and concentrated with Centricon YM filters (Amicon Co) by centrifugation for 6h at 4000 rpm. To determine the concentration, the purified protein was diluted into HEPES buffer and the absorbance at both 280 nm and 494 nm were measured. The absorption and fluorescence emission maxima of FITC-labeled proteins were 494 and 518 nm, respectively. The final protein concentration was calculated according to the formula: Protein concentration =

$([A_{280} - (A_{494} \times 0.30)] \times \text{dilution factor})/\epsilon$, where 0.30 is a correction factor (CF) and ϵ is the molar extinction coefficient of the protein at 280 nm. $\text{CF} = (A_{280} \text{ free dye}) / (A_{494} \text{ free dye}) = 0.30$ for FITC. The CF is included to compensate for absorption of the dye at 280 nm. The degree of labeling was calculated using $\epsilon = 68000 \text{ M}^{-1} \text{ cm}^{-1}$ for FITC. Labeled Max is termed Max-FITC. Max-FITC competed with unlabeled Max for DNA binding showing labeling did not perturb the protein binding (29).

DNA Oligonucleotides

The 16-bp oligonucleotide used in this study contained the sequence derived from the adenovirus major late promoter (MLP) containing the E-box (CACGTG). The DNA duplex contained the following sequence: 5'-TAGGCCACGTGACCGG-3' and its complementary strand. Complementary strands were annealed together to form duplex DNA. 20 μM of each DNA strand (labeled oligonucleotide and unlabeled complement) were dissolved in nuclease-free water, heated to 75–80 °C for 30 min, and allowed to cool at room temperature.

Stopped-Flow Anisotropy Measurements

Stopped-Flow fluorescence anisotropy experiments were performed with an OLIS RSM 1000 stopped-flow spectrophotometer equipped with a fluorescence polarization module. The dead time of the stopped-flow system, the age of the mixture when it is first available for measurement, is 1 ms. The instrument was configured in T-format, and emission was collected through 495 nm cut-on filters (provided by ORIEL Corporation, Stratford, CT) placed in front of each photomultiplier tube. A 450 W Xe arc lamp with a single grating monochromator was used. The excitation wavelength for FITC labeled Max protein ($^{\text{FITC}}\text{Max}$) was 490 nm. A temperature-controlled circulating water bath was used to maintain the temperature of the flow cell and solution reservoirs. After rapid mixing of samples, the time course of fluorescence anisotropy change was recorded by computer data acquisition. All measurements were carried out in titration buffer consisting of 20 mM HEPES/KOH, pH 7.6, 0.5 mM MgCl_2 , and 1 mM DTT containing 150 mM KCl. 1000 data points were collected per rapid-mix shot. For each experiment, the data from 6–10 shots were averaged to improve the signal to noise ratio. Each averaged set of stopped-flow data was then analyzed by nonlinear least squares fitting equations using Kaleidagraph (Synergy Software, West Palm Beach, FL). Data from the anisotropy experiments were fitted to the single and double exponential functions as described previously (35,36). Fitted curves correspond to the following single-exponential equation,

$$r(t) = r_0 + A e^{-k_{\text{obs}} t} \quad (1)$$

$r(t)$ is the anisotropy observed at any time t , r_0 is the anisotropy when the reaction reaches the equilibrium and A is the amplitude. k_{obs} is the observed first-order rate constant. The kinetic data for double exponential equation is

$$r(t) = r_0 + A_1 e^{-k_{\text{obs}1} t} + A_2 e^{-k_{\text{obs}2} t} \quad (2)$$

where A_1 and A_2 are the amplitudes ($r_t - r_0$) for the first and second components of double exponential reactions with observed rate constants $k_{\text{obs}1}$ and $k_{\text{obs}2}$, respectively. The residuals were measured by the differences between the calculated fit and the experimental data. All the reactions in this study were consistent with a single-exponential process. Arrhenius plots were constructed by using the observed rate constants at different temperatures according to the equation,

$$\ln k = (-E_a/RT) + \ln A \quad (3)$$

where k is the rate constant, E_a is the activation energy, R is the universal gas constant, T is the absolute temperature and A is the Arrhenius pre-exponential factor. Activation energies were calculated using the slopes of $\ln k$ versus $1/T$ (Kelvin).

Under pseudo-first order conditions, stopped-flow experiments were conducted using high concentrations of max, myc, mad and limiting concentrations of FITC labeled max to ensure that the bimolecular combination of ^{FITC}Max with unlabeled protein was pseudo first-order. The mechanisms considered involved a one and two step binding process (37).

For a one step model,

where M and D are the monomer and dimer protein, respectively; k_1 and k_{-1} are the forward and reverse rate constants, respectively. Under the pseudo first order condition, the observed rate constant is predicted to be a linear function of substrate concentration, i.e. $k_{obs} = k_1[C] + k_{-1}$.

For a two step model,

where M is the monomeric protein, D^* is an intermediate, and D is the final complex. The model involves the fast association of monomer protein followed by a slow change of conformation of the first association complex (D^*), to the stable complex (D), giving rise to fluorescence anisotropy change. The binding rates have the following relationship, assuming $k_2 \gg k_{-2}$ with the concentration of substrate: $1/k_{obs} = 1/k_2 + K_1/k_2 [C]$ as described previously (30,38), where k_{obs} is the observed first order rate constant, k_2 is the forward rate constant for the second step, K_1 is the equilibrium constant for the first step, and $[C]$ is the concentration of dimeric protein. A plot of $1/k_{obs}$ versus $1/[C]$ will give an intercept of $1/k_2$.

RESULTS

Kinetics of Heterodimer Formation are Faster than Homodimer Protein

The stopped-flow data for the dimerization of the transcription factors Max, Myc and Mad with FITC labeled Max were plotted as the anisotropy change *versus* time as shown in Figure 1. Stopped-flow kinetic traces were fitted to a single- and double-exponential equation using nonlinear regression analysis as described in Materials and Methods. The kinetic data showed that the binding of ^{FITC}Max·Myc ($k_2 = 90 \pm 4 \text{ s}^{-1}$) and ^{FITC}Max·Mad ($k_2 = 44 \pm 1.1 \text{ s}^{-1}$) was about 4.5- and 2.2-fold faster, respectively, than ^{FITC}Max·Max ($k_2 = 20 \pm 0.5 \text{ s}^{-1}$) binding (Figure 1 and Table 1). Treatment of the data using a double-exponential function did not improve the fit (Data not shown). The residuals representing the deviation between the calculated and experimental data (Figure 1, lower panel) indicate that the single-exponential function fits the points over the entire time course of measurements.

Under the pseudo-first order conditions, where unlabeled protein (Max, Myc or Mad) was in excess, the observed pseudo first-order rate constant is predicted to be a linear function of the concentration of ^{FITC}Max. To test this prediction, Myc·Max, Mad·Max and Max·Max dimerization experiments were performed over a concentration range of 0.2–2 μ M, 4–40 fold excess. In all these experiments, the concentration of the FITC-labeled Max was kept constant at 50 nM. According to K_d values (29) of Myc, Mad and Max interactions, 90% of the Max form dimer with Myc and Mad. Figure 2 shows the rate constants of the Max·Max, Myc·Max and Mad·Max interactions at different concentrations. The Max, Myc and Mad dimerization rates were found to be nearly concentration independent suggesting conformational changes are rate limiting.

For a model involving a conformational change, explained in Materials and Methods, the binding rates have a relationship with the concentration of substrate as described previously (30,38), $1/k_{obs} = 1/k_2 + K_1/k_2[C]$, where k_{obs} is the observed first order rate constant, k_2 is the forward rate constant for the second step, K_1 is the equilibrium constant for the first step, and $[C]$ is the concentration of the unlabeled protein Max, Myc or Mad. The $1/k_{obs}$ versus $1/[C]$ plot shown in Figure 2 for Max·Max, Myc·Max and Mad·Max dimerization confirm the predicted linear relationship. k_2 values, obtained from the intercept, were found to be 20 ± 1.1 , 91 ± 2.3 and 44.3 ± 1.4 s⁻¹ for Max·Max, Myc·Max and Mad·Max dimerization, respectively.

Rate constants for all three protein-protein interactions, Max, Myc and Mad binding to ^{FITC}Max, at different temperatures are shown in Table 1. The stopped-flow kinetic data revealed that at all four temperatures Myc·Max dimerization had the highest rate constants compared to the other two transcription factor dimerizations. Myc·Max interaction had a 4.0 to 6 times higher rate constant than Max·Max homodimerization and 2.0 times higher than Max·Mad dimerization. The values of k_2 for Max, Myc and Mad protein binding to ^{FITC}Max at different temperatures were calculated from the data sets collected at each temperature (Table 1) as a representative plot shown in Figure 1.

The rate constants in Table 1 were used to construct Arrhenius plots (Figure 3) according to Equation 3. The activation energies were obtained from the slope of the linear fit of $\ln k$ versus $1/T$ plots. Our results show that the activation energy for the heterodimer Max·Myc and Max·Mad is ~2.0 and 1.4-fold lower than for the homodimer Max·Max, respectively. The overall lower activation energy for heterodimer binding suggests a number of more favorable hydrogen bonds are formed in the transition state.

Homodimer Protein binds to DNA Faster than Heterodimer Protein

To determine the DNA binding rate constants of homodimer Max·Max and heterodimers Myc·Max and Mad·Max, stopped-flow anisotropy experiments were performed using 16-mer 5' FITC labeled DNA E-Box oligonucleotide. In all these kinetic studies, the final concentration of the 5' FITC labeled 16-mer DNA oligonucleotide was kept constant at 50 nM. Varying concentrations of the unlabeled Max·Max, Myc·Max and Mad·Max protein dimers were injected into the mixing chamber of the stopped-flow instrument. 0.2 μ M of Max was pre-incubated with 2 μ M of Myc or Mad protein at room temperature for 30 min prior to the reaction with the DNA E-Box. K_d values (29) for the protein-protein interactions were used to calculate protein dimer formation. 91% of the Myc·Max and Mad·Max were in the dimer form at 200 nM of the protein mixing concentration. The stopped-flow data for the binding of FITC labeled 16-mer DNA with Max·Max, Max·Myc and Max·Mad protein complexes were plotted as the anisotropy versus time as shown in Figure 4. The changes in the anisotropy as a function of time for the three different protein dimers binding to E-Box DNA were recorded and data were fitted using nonlinear regression analysis as a single and double-exponential (Equation 1 and 2). The binding of Max·Max ($k_{obs} = 132 \pm 7.4$ s⁻¹) showed about 2-fold higher rate constant than Max·Mad ($k_{obs} = 78 \pm 2$ s⁻¹) for binding to DNA E-Box (Figure 4). Myc·Max binding

rate constants were similar to Max·Max. Treatment of the data using a double-exponential function did not improve the fit (Data not shown).

To determine whether protein dimer-DNA rates are concentration dependent, $1/k_{\text{obs}}$ versus $1/[C]$ graph was plotted (Figure 5). The kinetic plots revealed that the interactions between the protein dimers Max·Max, Myc·Max and Mad·Max with the DNA E-box are concentration independent. From the intercept of $1/k_{\text{obs}}$ Vs $1/[C]$, k_2 was found to 133 ± 5 , 122 ± 4.3 and $82 \pm 4 \text{ s}^{-1}$ for Max·Max-DNA, Myc·Max-DNA and Mad·Max-DNA interactions, respectively.

The interactions between Max·Max, Myc·Max and Mad·Max protein dimers and FITC labeled DNA E-box oligonucleotide were also investigated at different temperatures. The effects of the temperature on the rate constants of these protein dimer-DNA reactions are shown in Table 2. The final concentration of the protein dimers and DNA E-Box in the mixing chamber of the stopped-flow instrument were $0.2 \mu\text{M}$ (each) and 50 nM , respectively. The rate for the reaction of Max·Max and Myc·Max dimers is significantly faster than that of Mad·Max with DNA E-Box. From the temperature dependence of the rate constants of these dimer-DNA interactions, Arrhenius plots (Figure 6) were obtained and activation energies, E_a , were evaluated from their slopes. Analysis of the Arrhenius plots show that the activation energies for Max·Max-DNA ($E_a = 10.5 \pm 0.5 \text{ kJ/mol}$) and Max·Myc-DNA ($E_a = 12 \pm 0.4 \text{ kJ/mol}$) are very similar. The activation energy for the interaction of Max·Mad dimer ($E_a = 20 \pm 0.3 \text{ kJ/mol}$) is about 2-fold higher than for Max·Max and Max·Myc dimers with DNA E-Box (Table 2).

Addition of Second Monomer to the Monomer-DNA Complex Lower the Rate

In order to compare the monomer/dimer-DNA kinetics, we further studied the monomer-DNA kinetics. Max is the only protein among the three b/HLH/z family transcription factors Myc, Max and Mad, which has a significant affinity for DNA. The other two proteins, Myc and Mad, in the form of monomers, have a very low affinity for DNA (15). They form homodimers weakly and bind DNA poorly. Therefore, binding of monomeric Myc and Mad to DNA can be neglected. To avoid the DNA binding of dimeric Max proteins, we determined the most suitable experimental conditions using the previously published equilibrium data (26,29). To ensure that 90% of Max protein is in the monomeric form not dimeric, the required concentration of Max protein was calculated using the K_d value (29) for monomer-dimer equilibrium. To determine kinetic rates, 50 nM Max protein and $1 \mu\text{M}$ 16-mer DNA oligonucleotides were mixed in the stopped-flow mixing chamber and association rate constant were observed as shown in Figure 7. The association rate constant for binding of monomeric Max protein to DNA E-box was $158 \pm 4.4 \text{ s}^{-1}$. Binding of the second monomer (Myc) to form a heterodimer-DNA complex may serve to aid in selection of DNA. The association rate constant for Max-DNA complex with Max, Myc and Mad were $138 \pm 5 \text{ s}^{-1}$, $126 \pm 4.1 \text{ s}^{-1}$ and $101 \pm 4 \text{ s}^{-1}$, respectively. Monomer-DNA (Max-DNA, $k_2 = 158 \pm 4.4 \text{ s}^{-1}$) has a faster rate than dimer-DNA kinetics.

Figure 8 shows the pathway for the formation of b/HLH/Z transcription factors (Max, Myc and Mad) - DNA complex via a dimer and monomer pathway. Dimer pathway, first proteins form dimers, which then bind to the DNA and form a dimer-DNA complex. In the monomer pathway, first a monomer binds to DNA and forms a monomer-DNA complex, followed by recruitment of the second monomer to form the final complex. Equilibrium 1 followed by equilibrium 2 is the dimer pathway. Equilibria 3 and 4 represent the monomer pathway. The monomer and dimer pathways are energetically equivalent (thermodynamic cycle) and preference for one or the other is kinetic. Our kinetic data is very much in agreement with the proposed monomer-DNA binding pathway. Here we provide kinetic constants and show that dimer formation is the rate-limiting step in the dimer pathway whereas both steps of the monomer pathway are rapid and have comparable rates.

DISCUSSION

In this study, we provide details of protein dimer-DNA kinetics, which form complexes with DNA via the monomer pathway. Previously, proteins belonging to b/Z (39,40) and b/HLH (41), nuclear hormone receptor (22) families, Arc (42), and LexA (43) repressors have been shown to bind DNA using the monomer pathway.

Stopped-flow fluorescence resonance energy transfer (FRET) studies conducted on cFos and cJun by Kohler and Schepartz (39) showed that although the dimerization of these proteins occurred rapidly in the absence of DNA, the rate of dimerization was enhanced in the presence of DNA. Their explanation of why the DNA binding is accelerated is based on electrostatic interactions between the proteins and DNA. Charge-charge interactions between the negatively charged DNA and positively charged proteins are the main attractive forces, which form the monomer-DNA interface (44). For Coulombic electrostatic interactions, the force between two molecules of opposite charges is proportional to the inverse of the separation distance squared. Thus, in the case of basic protein monomers and acidic DNA, one expects an accelerated binding. Our study showed that the rate of protein dimerization (step 1 in Figure 8) is limiting the overall rate of the dimer pathway (step 1 followed by step 2 in Figure 8). Therefore, the monomer pathway provides a faster route to the final complex. Another advantage of the monomer pathway lies in the fact that unfolded proteins (monomeric Max) have a bigger capture radius than folded proteins (dimeric b/HLH/Z proteins) (44). Because of this larger capture radius, monomeric Max recognizes DNA with an increased rate. Studies showed that binding kinetics of monomeric Fos and monomeric Jun to DNA provides a better fit to the experimental data than the dimer pathway (39). In a previous work published by Park et al (45), the association kinetic constants of the interaction of truncated Max·Max and Myc·Max dimers with DNA were determined. Their results are also in good agreement with ours in terms of similarity of the rate constants of Myc·Max and Max·Max binding to DNA. They found that the Max·Max dimer recognized DNA at almost the same rate as Myc·Max, which was also the case in our present study.

Previously the binding and thermodynamic stability and specific DNA binding to Myc, Max and Mad family (26,29,46) has been determined. In order to fully understand the forces involved in protein-DNA recognition, we report the pre-steady state kinetic results on the interactions of the b/HLH/Z family transcription factors, Max, Myc and Mad with each other and also with DNA using stopped-flow anisotropy. The concentration dependencies of the observed rates of these processes correspond with a two-step reaction model in which initial fast formation of dimer-DNA complex is followed by a conformational change of the complex in a rather fast step, but with a measurable rate.

In the case of formation of Myc·Max and Mad·Max heterodimers, the activation energy was lower compared to Max·Max homodimer. This reduced temperature dependence suggests that the heterodimer interaction is accelerated and a substantially lower energy barrier is provided. The lower activation energy of the Max·Myc and Max·Mad dimerization suggests an intermediate that more easily achieves a stable conformation. Binding of Myc to Max monomer has higher rate constants at all temperatures than Max·Max and Mad·Max formation, suggesting that Myc·Max complex is able to more rapidly change conformation.

It was observed that dimer-DNA interaction was concentration independent. Binding of dimeric transcription factors to a 16-mer oligonucleotide exhibits two kinetic phases: initial rapid phase and a rather fast, concentration independent phase. The reaction is interpreted as the concentration-dependent initial complex, which rearranges in a concentration-independent step to a thermodynamically stable complex. The rate for the interaction of the dimeric proteins with E-Box DNA is faster than the rate of the formation of the protein dimers, at all

temperatures. The rates for the formation of Myc·Max-DNA and Max·Max-DNA complexes were higher than the Mad·Max-DNA complex. The Arrhenius activation energies for the binding of Max·Max and Myc·Max is about 2-fold lower as compared to Max·Mad binding to DNA E-Box.

The conclusions of a similar study by Kohler and Schepartz (39) suggest that assembly of the dimeric transcription factors Jun-Fos and specific DNA follows the monomer pathway. However, Kohler and Schepartz preferred an experimental condition, in which the dimer-DNA complex was formed from monomeric Jun, monomeric Fos and DNA. However, in cellular environment there are monomeric and also dimeric proteins presented which may compete for DNA. In such a case, the ratio of monomeric proteins to dimeric ones may be a crucial factor for determining the choice of the pathway. It is important to recognize that the assembly of transcription factor-DNA complexes *in vivo* is more intricate than *in vitro*. In the cellular environment, the transcription factors discriminate between specific and non-specific DNA as well as a large excess of other proteins with which the transcription factors have to compete for DNA.

The most commonly discussed theory for DNA-binding proteins to recognize their specific sequences is “facilitated diffusion” (47). According to this theory, proteins find their target by sliding along the DNA. This is one-dimensional diffusion during which the protein remains in contact with the DNA for long enough periods so that appreciable diffusive motion occurs in either direction along the chain contour. Since the unspecific DNA binding of a monomeric protein is weak compared to a dimeric one due to more interactions between the DNA, it may slide faster along the DNA. The diffusion rate of a monomeric protein may be more rapid because it is sterically less hindered. Therefore one of the reasons why the monomer pathway is preferred over the dimer pathway may be the fast diffusion rate of the monomeric transcription factor along the DNA.

Another factor affecting the rate of target recognition is the stability of the protein-DNA complex. Accessory proteins may influence the stability of these complexes by binding to monomeric or dimeric transcription factors. Hence, the rate of DNA binding through a monomer or dimer pathway may differ depending on whether such accessory proteins bind to the monomeric or dimeric transcription factors. Future experiments will explore the effect of accessory proteins on the stability of these complexes and the diffusion rates of monomeric and dimeric transcription factors along the DNA.

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Figure 1.

Stopped-flow kinetic binding measurements of Max, Myc and Mad to FITC labeled Max protein. Representative kinetic data show the time dependent increase in anisotropy after mixing 50 nM ^{FITC}Max (final concentration) with 0.2 μ M unlabeled (final concentration) of (A) Myc, (B) Mad and (C) Max at 21°C. Residuals for the fits are shown in the lower panels. The experimental conditions are described under “Experimental Procedures”.

Figure 2. Kinetic plots of $1/k_{\text{obs}}$ versus $1/[C]$ for the interaction of 50 nM FITCMax with varying concentrations of Max (—○—), Myc (—●—) and Mad (—△—) protein. The rate constant k_2 was obtained as the reciprocal of the y-intercept.

Figure 3.

Arrhenius plots for the interaction of Max, Myc and Mad with ^{FITC}Max. The rate constant values for Max (—○—), Myc (—●—) and Mad (—△—) protein with ^{FITC}Max protein at different temperatures were used to construct an Arrhenius plot according to equation 5. The activation energy was calculated from the slope of the fitted linear plot of $\ln k$ versus T^{-1} (Kelvin).

Figure 4.

Stopped-flow kinetic binding measurements of Max·Max, Myc·Max and Mad·Max protein dimer to FITC labeled 16-mer DNA E-Box. Representative kinetic data show the time dependent increase in anisotropy after rapid mixing of 50 nM ^{FITC}DNA E-Box (final concentration) with 0.2 μM unlabeled (final concentration) of (A) Max·Max (B) Max·Myc and (C) Max·Mad at 21°C. Residuals for the fits are shown in the lower panels. The experimental conditions are described under “Experimental Procedures”.

Figure 5. Kinetic plots of $1/k_{\text{obs}}$ versus $1/[C]$ for the interaction of 50 nM FITC -DNA E-Box with varying concentrations of Max·Max ($\circ$), Max·Myc ($\bullet$) and Max·Mad ($\triangle$) dimmer protein. The rate constant k_2 was obtained as the reciprocal of the y-intercept.

Figure 6. Arrhenius plots for the interaction of ^{FITC}DNA E-Box with Max·Max, Max·Myc and Max·Mad dimer protein. The rate constant values for Max·Max (—○—), Max·Myc (—●—) and Max·Mad (—△—) protein at different temperatures were used to construct an Arrhenius plot according to equation 5. The activation energy was calculated from the slope of the fitted linear plot of $\ln k$ versus T^{-1} (Kelvin).

Figure 7.

Kinetic measurements for the binding of monomer protein to DNA E-box. Data shows the time dependent increase in anisotropy after rapid mixing (A) 50 nM ^{FITC}Max (final concentration) with 1 μ M DNA E-box (final concentration), (B) Max-DNA complex with 1 μ M Max, (C) Max-DNA complex with 1 μ M Myc, and (D) Max-DNA complex with 1 μ M Mad, respectively, at 21°C.

Figure 8.

Schematic diagram of the Myc, Max, and DNA E-box interactions. The model shows the two pathways k1, k2 and k3, k4 for the formation of Myc•Max•DNA complex. The k values at different temperatures are shown in Table 1 and 2.

Table 1

Kinetic binding constants for the interaction of transcription factor Max, Myc and Mad with FITC labeled Max.

Temp	Association Rate Constants, k_{obs} (s^{-1})		
	Max·Max	Max·Myc	Max·Mad
13 °C	11.1 ± 0.4	67 ± 2	32.2 ± 0.7
21 °C	20 ± 0.5	90 ± 4	44 ± 1.1
27 °C	22.1 ± 0.4	109 ± 4.0	55.4 ± 1.5
32 °C	30 ± 0.7	118 ± 7	65 ± 5
E_a (kJ/mol)	40 ± 0.2	20.4 ± 0.8	29 ± 0.6

Table 2

Kinetic binding constants for the interaction of transcription factor Max·Max, Max·Myc and Max·Mad with DNA E-Box.

Temp	Association Rate Constants, k_{obs} (s^{-1})		
	Max·Max-DNA	Max·Myc-DNA	Max·Mad-DNA
13 °C	116 ± 5.0	103 ± 5	60.4 ± 6
21 °C	132 ± 7.4	118 ± 2.4	78 ± 2
27 °C	147 ± 8.0	127 ± 8.4	86 ± 3
32 °C	151 ± 5	144 ± 6.2	98 ± 1.5
E_a (kJ/mol)	10.5 ± 0.5	12.0 ± 0.4	20 ± 0.3