The α and β Subunits of the GA-binding Protein Form a Stable Heterodimer in Solution

REVISED MODEL OF HETEROTETRAMERIC COMPLEX ASSEMBLY

Yuri Chinenov‡, Michael Henzl, and Mark E. Martin§

From the Department of Biochemistry, University of Missouri at Columbia, Columbia, Missouri 65212

We have studied the assembly of GA-binding protein (GABP) in solution and established the role of DNA in the assembly of the transcriptionally active GABPαβ2 heterotetrameric complex. GABP binds DNA containing a single PEA3/Ets-binding site (PEA3/EBS) exclusively as the αβ heterodimer complex, but readily binds as the GABPαβ2 heterotetramer complex on DNA containing two PEA3/EBSs. Positioning of the PEA3/EBSs on the same face of the DNA helix stabilizes heterotetramer complex binding. These observations suggest that GABPαβ heterodimers are the predominant molecular species in solution and that DNA containing two PEA3/EBSs promotes formation of the GABPαβ2 heterotetrameric complex. We analyzed the assembly of GABPαβ2 heteromeric complexes in solution by analytical ultracentrifugation. GABPα exists as a monomer in solution while GABPβ exists in a monomer-dimer equilibrium (Kd = 1.8 ± 0.27 μM). In equimolar mixtures of the two subunits, GABPα and GABPβ formed a stable heterodimer, with no heterotetramer complex detected. Thus, GABP exists in solution as the heterodimer previously shown to be a weak transcriptional activator. Assembly of the transcriptionally active GABPαβ2 heterotetramer complex requires the presence of specific DNA containing at least two PEA3/EBSs.

The GA-binding protein (GABP) is a heteromeric transcription factor that binds to GA-rich sequences ([A/C]GGAAG) in DNA, and contains two unrelated subunits belonging to the Ets (GABPα) and the Notch-Ankyrin repeat (GABPβ) families of proteins (1–5). GABP has been implicated in the regulation of several eukaryotic genes encoding proteins involved in oxidative phosphorylation (cytochrome oxidase subunits IV, V, and VII and ATP synthase β subunit) (6–12) and the immune and inflammatory response (CD18, interleukin-2, γc chain of interleukin receptors) (13–17). Recently, an important role for GABP in the regulation of mitochondrial biogenesis in brown adipose tissue was demonstrated (12).

The GABP-binding site first identified in the herpes simplex virus immediate early promoter contains two tandemly arranged PEA3/Ets-binding sites (EBS) (1, 18, 19). Early analysis of GABP binding to the herpes simplex virus immediate early promoter indicated that GABP forms predominantly a heterotetrameric complex composed of two α and two β subunits (1, 2). The results of gel filtration and preparative ultracentrifugation of highly purified recombinant GABPα and GABPβ proteins were further interpreted to support a model depicting GABP as a stable heterotetrameric complex in solution, suggesting that this complex binds as a single unit to DNA (1, 2).

The ability to form the heterotetrameric complex has been shown to be necessary for GABP-dependent transcription (20–22). Thus, modulation of GABP tetramer formation represents a potentially important means of regulating GABP-dependent transcription.

We have previously shown that GABP-dependent transcription initiator activity required two PEA3/EBSs, and the highest activity was achieved with two sites positioned on the same face of the DNA helix (23–25). Since the ability to activate transcription was previously shown to be dependent on heterotetramer complex formation, these results favored the stable GABP heterotetramer complex model. However, electrophoretic mobility shift assays (EMSA) with DNA probes containing only a single PEA3/EBS detected a complex consistent with the mobility of the GABPαβ heterodimer (23, 26, 27). In contrast, a probe with two PEA3/EBSs formed two complexes, one co-migrating with the GABPαβ complex observed on a single PEA3/EBS and a slower migrating complex identified as GABPαβ2 heterotetramer. These observations are consistent with the notion that GABP binds to a single PEA3/EBS only as a heterodimer, and requires two or more PEA3/EBSs to bind DNA as a heterotetramer. Therefore, if GABP forms a stable heterotetrameric complex in solution, then dissociation of this complex would be required upon binding to a single PEA3/EBS. Alternatively, GABP may form a stable heterodimer, or may exist in heterodimer-heterotetramer equilibrium that, at concentrations typically found in EMSA assays, favors the GABPαβ heterodimer. Therefore, prior interaction of a GABPαβ heterodimer complex with DNA containing two PEA3/EBSs may be required for successful assembly of a transcriptionally competent GABPαβ2 heterotetramer.

To address the mechanism of GABP assembly and the role of DNA binding in this process, we have utilized EMSAs and analytical ultracentrifugation. In this report we show that GABP exists exclusively as a stable heterodimeric complex (GABPαβ). Even at exceedingly high concentrations little or no significant quantities of GABP heterotetramer complex is observed. These observations support the hypothesis that GABP exists as a stable heterodimer in solution which assembles into...
the heterotetrameric complex upon binding to target DNA containing two PEA3/EBSs. We further demonstrate that heterotetramer DNA binding is stabilized by positioning the two PEA3/EBSs on the same face of the DNA helix, and that the two motifs can be separated by up to three helical turns. These results are consistent with our earlier results demonstrating that GAPB-dependent initiators were most efficient when two PEA3/EBSs were positioned on the same face of the DNA helix, and confirms the importance of the GAPB heterotetramer complex in transcription initiation and activator activities of this important regulatory factor (23).

EXPERIMENTAL PROCEDURES

Materials—DNA-modifying enzymes were purchased from New England Biolabs. Nickel-nitrilotriacetic acid-agarose was purchased from Qiagen. Talon Metal Affinity Resin for cobalt immobilized metal affinity chromatography was purchased from Clontech. DNA oligonucleotides were synthesized by the University of Missouri DNA Core Facility on an Applied Biosystems DNA Synthesizer, Model 380B. [α-32P]dGTP, [α-32P]dCTP, and [γ-32P]ATP were purchased from NEN Life Science Products Inc. All other reagents were obtained from Sigma or Fisher Scientific.

Cloning and Expression of Recombinant Proteins in Escherichia coli—DNAs encoding GAPB and GAPB proteins were amplified by polymerase chain reaction from cDNAs kindly provided by C. C. Thompson and cloned into pTET15B (Novagen) as described previously (26). The individual recombinant (rGAPB) His6-tagged proteins (Fig. 1A) were expressed in E. coli BL21 strain and recovered from cell extracts by nickel chelating or cobalt immobilized affinity chromatography under denaturing conditions in the presence of 6 M urea. rGAPB and rGAPB proteins were precipitated with ammonium sulfate at 20 and 14% saturation, respectively. Precipitated material was resuspended in 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM potassium chloride, 6 mM urea, and 5 mM diethiothreitol and subjected to several steps of dialysis in the same buffer with progressively lower concentrations of urea (5M, 3 M, etc.). The extinction coefficients of GAPB and GAPB proteins were precipitated with ammonium sulfate at 20 and 14% saturation, respectively. Precipitated material was resuspended in 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM potassium chloride, 6 mM urea, and 5 mM diethiothreitol and subjected to several steps of dialysis in the same buffer with progressively lower concentrations of urea (5 M, 3 M, etc.). The extinction coefficients of GAPB and GAPB proteins were calculated from their amino acid compositions. The molecular weights of the first, second, and monomeric species, respectively, at a reference point. M1, M2, and Mm are the molecular weights of the first, second, and monomeric species, respectively. The angular velocity, ω, was calculated from the rotor speed. The value for partial volume, F, at 20 °C was calculated from amino acid composition using SEDNTERP software. R is the universal gas constant. T is the absolute temperature equal to 293.15 K. The parameter C is the baseline that was either determined from the absorbance near the meniscus after sedimentation of the samples at 20,000 rpm for 12 h or from global least-square analysis.

To account for nonspecific aggregation at high protein concentrations or for deviations from stoichiometry in multi-component mixtures, a third term was introduced into equation III, producing Equation 4.

RESULTS

GABP Requires Two PEA3/EBSs for Heterotetramer Complex Formation on DNA—The GAPB-binding site originally identified in the HSV IE promoter contained two tandemly arranged PEA3/EBSs (1, 2, 18). When two PEA3/EBSs (dPEA3-0) are present on the target DNA, approximately equal amounts of both heterodimeric and heterotetrameric complexes are readily observed (Fig. 1B). However, on a probe containing only a single PEA3/EBS (PEA324), GAPB forms exclusively the heterodimeric complex (Fig. 1B). No heterotetramer complex was observed on the PEA324 probe containing a single PEA3/EBS even at 10-fold higher GABP protein concentrations. These results indicate that at the concentration used in these EMSA experiments (10 nm), GABP requires two PEA3/EBSs to efficiently assemble into a heterotetrameric complex on DNA.

Deletion of the Amino Terminus of GABPα Enhances Heterotetramer Complex Formation on DNA—Previous studies indicated that an NH2-terminal truncation mutant of the GABPα protein (GABPα-Q) exhibited enhanced heterotetramer complex DNA in the presence of GABPβ protein (26, 27). GABPα-Q protein (Fig. 1A) readily forms both heterodimeric and heterotetrameric complexes with GAPBβ protein on DNA containing a single PEA3/EBS (Fig. 1B). When two PEA3/EBSs are present on the target DNA, only the heterotetrameric complex formed in a Beckman Optima XL-I ultracentrifuge equipped with AN50ti rotor (Beckman Instruments, Palo Alto, CA). Six-channel cells with 12-mm optical path length were used. Data were collected at 20 °C at rotor speeds between 6,000 and 28,000 rpm. Sample loading concentrations ranged between 2 and 13 μM in 50 mM phosphate buffer, pH 7.4, containing 100 mM KCl, 10 mM glycine, and 1 mM diethiothreitol. The distribution of solutes in the cell was monitored by absorbance at 280 nm. The resulting distributions at equilibrium were subjected to global least-square analysis employing several models: a single exponential (monomeric ideal, Equation 1), two exponential (dimeric ideal, Equation 2), or two exponential (associative, Equation 3) (28) using the program Origin 5.0 (MicroCal Software, Inc., MA).

\[
C(r) = C_{10} \times \exp \left( \frac{\alpha^2 \times (1 - \frac{v}{r}) \times M_1 \times (r^2 - r_0^2)}{2 RT} \right) + C_a 
\]

\[
C(r) = C_{10} \times \exp \left( \frac{\alpha^2 \times (1 - \frac{v}{r}) \times M_1 \times (r^2 - r_0^2)}{2 RT} \right) + C_{20} 
\]

\[
C(r) = C_a \times \exp \left( \frac{\alpha^2 \times (1 - \frac{v}{r}) \times M_1 \times (r^2 - r_0^2)}{2 RT} \right) + C_m \times K 
\]

Where \(C_{10}, C_{20},\) and \(C_m\) are the absorbance of the first, second, and monomeric species, respectively, at a reference point. \(M_1, M_2,\) and \(M_m\) are the molecular weights of the first, second, and monomeric species, respectively. The angular velocity, \(\omega,\) was calculated from the rotor speed. The value for partial volume, \(F,\) at 20 °C was calculated from amino acid composition using SEDNTERP software. \(R\) is the universal gas constant. \(T\) is the absolute temperature equal to 293.15 K. The parameter \(C\) is the baseline that was either determined from the absorbance near the meniscus after sedimentation of the samples at 20,000 rpm for 12 h or from global least-square analysis.

To account for nonspecific aggregation at high protein concentrations or for deviations from stoichiometry in multi-component mixtures, a third term was introduced into equation III, producing Equation 4.

\[
C(r) = C_{20} \times \exp \left( \frac{\alpha^2 \times (1 - \frac{v}{r}) \times M_2 \times (r^2 - r_0^2)}{2 RT} \right) + C_m \times K 
\]

\[
C(r) = C_a \times \exp \left( \frac{\alpha^2 \times (1 - \frac{v}{r}) \times M_1 \times (r^2 - r_0^2)}{2 RT} \right) + C_m \times K 
\]

\[
C(r) = C_a \times \exp \left( \frac{\alpha^2 \times (1 - \frac{v}{r}) \times M_1 \times (r^2 - r_0^2)}{2 RT} \right) + C_m \times K 
\]

\[
C(r) = C_a \times \exp \left( \frac{\alpha^2 \times (1 - \frac{v}{r}) \times M_1 \times (r^2 - r_0^2)}{2 RT} \right) + C_m \times K 
\]
is observed. Thus, enhanced GABP heterotetrameric complex DNA binding is achieved by deletion of the NH2-terminal two thirds of the GABPα protein. Enhanced DNA binding of the heterotetramer complex requires the COOH-terminal leucine zipper-like domain in GABPβ. Deletion of this region in the GABPβΔ334 mutant abolishes the formation of detectable heterodimeric complex with either full-length GABPβ or GABPαα proteins on probes containing either one or two PEA3/EBSs.

**GABP Heterotetramer Complex DNA Binding Is More Stable When Two PEA3/EBSs Are Positioned on the Same Face of the DNA Helix**—We have previously shown that two PEA3/EBSs function as a GABP-dependent initiator element, and that maximal initiator activity occurs on templates containing two PEA3/EBSs on the same face of the DNA helix (23). In addition, GABP activation has been shown previously to depend on the ability of GABP to form the heterotetrameric complex (20–22). Therefore, we sought to determine whether the preference of GABP-dependent initiator elements for templates containing two PEA3/EBSs on the same face of the DNA helix correlates with the ability of GABP heterotetrameric complex to form on these initiator elements. We performed EMSA analysis of GABP binding to DNA probes containing two PEA3/EBSs separated by a 0, 10-, 16-, 22-, or 26-base pair linker DNA, corresponding to 0.5, 1.5, 2.0, 2.5, and 3.0 helical turns between PEA3/EBS elements. Approximately equivalent amounts of heterodimeric complex is not affected by the helical spacing between two PEA3/EBSs.

Analysis of heterotetrameric complex DNA binding revealed that GABP heterotetrameric complexes bound to DNA containing two PEA3/EBSs on the same face of the DNA helix were significantly more stable than complexes bound to DNA containing two PEA3/EBSs on the opposite sides of the DNA helix.
GABP heterotetramer complexes bound to radiolabeled probes were challenged with an excess of unlabeled competitor DNA and the rate of heterotetramer complex decay measured over time. Heterotetramer complexes bound to probes containing two PEA3/EBSs separated by 2.0 and 3.0 helical turns were substantially more resistant to challenge by the unlabeled competitor DNA than were complexes bound to probes containing two PEA3/EBSs separated by 0.5, 1.5, and 2.5 helical turns. Heterotetramer complexes bound to all probes were substantially more stable than the heterodimer complex bound to DNA containing a single PEA3/EBS. Thus, the ability to assemble into a heterotetrameric complex during DNA binding is independent of helical spacing or distance (up to 4 helical turns), but complex stability is enhanced when two PEA3/EBSs are positioned on the same face of the DNA helix.

Fig. 3. Sedimentation equilibrium analysis of individual GABPα, GABPαcQ, GABPβ, and GABPβ334 proteins. Analysis was performed at 20 °C in a Beckman Model XL-I ultracentrifuge at speeds of 6,000, 9,000, and 12,000 rpm (GABPα), 12,000, 16,000, and 20,000 rpm (GABPαcQ), or 9,000, 12,000, and 15,000 rpm (GABPβ, and GABPβ334), and at initial concentrations ranging between 0.1 and 0.7 A280 nm. Concentration distributions were subjected to global least square analysis using Origin 5.0 software (Microcal). The quality of fit is illustrated by the random distribution of residuals, i.e., the differences between calculated and observed values. A, the concentration distributions for the GABPα protein were treated using an ideal single-species model (Equation 1, “Experimental Procedures”), with the molecular weight equal to that of the His6-tagged GABPα protein. B, the concentration distributions for the GABPαcQ protein were treated using an ideal single-species model (Equation 1, “Experimental Procedures”) with a molecular weight equal to that of His6-tagged GABPαcQ. C, the concentration distributions for GABPβ were fitted to a two-species associative model (Equation 3, “Experimental Procedures”) with the molecular weight of the monomer equal to that of His6-tagged GABPβ. D, the concentration distributions for GABPβ334 were fitted to a two-species ideal model (Equation 2, “Experimental Procedures”) with the molecular weight of the first species equal to that of His6-tagged GABPβ334. The molecular mass of the second species was 1,417.503 ± 69.435 kDa.
relating helical spacing between PEA3/EBSs with GABP initiator activity.

**GABPα and GABPα,Q Proteins Are Monomeric in Solution—**

The association states of GABPα, GABPβ, and the various mutant proteins (Fig. 1) were analyzed by sedimentation equilibrium. The distribution of GABPα (Fig. 3A) is satisfactorily described by a single species model ("Experimental Procedures," Equation 1), in which the molecular weight is set to the calculated value of 53,500 for the His6-tagged GABPα protein. We attribute the discrepancies between the calculated and experimental values observed at the highest protein concentration and rotor speeds, to nonspecific aggregation of GABPα. These data suggest that GABPα exists as a monomeric species under our experimental conditions. GABPα,Q protein behaved similarly (Fig. 4A), although no aggregation was observed. The radial distribution for GABPα,Q (Fig. 4B) is accommodated by an ideal single species model in which the molecular mass is fixed at 18,551 Da, the calculated molecular mass of His6-tagged GABPα,Q. In contrast to the full-length GABPα protein, we observe no indication of higher molecular weight aggregates.

**GABPβ Weakly Self-associates in Solution Through the COOH-terminal Leucine Zipper-like Domain—**

In contrast to GABPα, we see evidence for dimerization, albeit weak, of GABPβ. The distribution of GABPβ in sedimentation equilibrium experiments is consistent with a monomer-dimer equilibrium associative model (Equation 3) (Fig. 3C). The mass of the monomeric species was fixed at 43,422 Da, equal to that of His6-tagged GABPβ, and the dissociation constant was treated as a variable parameter. The optimal value for the homodimer dissociation constant, $K_{dβ}$, was determined to be $1.8 \pm 0.27 \mu M$, indicating that GABPβ associates only weakly under these conditions. At physiologically relevant concentrations (0.1–10 nM), GABPβ would be almost exclusively monomeric.

Deletion of the COOH-terminal portion of GABPβ (GABPβ$_{334}$), which includes the leucine zipper-like domain,
eliminates the tendency to self-associate. The radial distribution of GABPβ<sub>334</sub> is consistent with an ideal two-species model (Equation 2) with the molecular mass of the first species equal to that of His<sub>6</sub>-tagged GABPβ<sub>334</sub> protein (37,691 Da), and the molecular mass of the second species equal to 1,417,503 ± 69,435 Da (Fig. 3D). GABPβ<sub>334</sub> protein tends to precipitate at high protein concentrations suggesting that the second high molecular weight species may be attributed to nonspecific aggregation of the GABPβ<sub>334</sub> protein at high concentrations. Consistent with this assumption, the molecular mass of the second species varied from preparation to preparation but was consistently higher than 600 kDa. These results are consistent with the previously proposed role of the COOH-terminal leucine zipper-like domain of GABPβ in self-association (1–3, 20, 29).

**GABP Exists Predominantly as a Heterodimer in Solution in the Absence of DNA**—The virtual absence of heterotetrameric GABP bound to a single PEA3/EBS in EMSAs suggests that any heterotetrameric complex formed in solution must have a low affinity for a single PEA3/EBS. Alternatively, GABP heterodimers may have very little tendency to assemble into the heterotetrameric complex on DNA containing a single PEA3/EBS. To distinguish between these possibilities, we performed analytical ultracentrifugation to determine the relative amounts of GABP heterodimer and heterotetramer complexes in solution in the absence of DNA binding.

When stoichiometric amounts of GABP<sub>a</sub> and GABP<sub>b</sub> are combined and centrifuged to equilibrium, the resulting radial distribution is consistent with exclusive heterodimer formation. Optimal agreement between the calculated and observed values was obtained with an ideal two species model (Equation 2), in which the molecular mass for the major species was fixed at 61,525 Da (the sum of molecular masses of GABP<sub>a</sub> and GABP<sub>b</sub>). The mass for the second species was allowed to vary and converged to 619,573 ± 13,597 Da (Fig. 4A). The presence of this material, which accounted for less than 5% of the total protein, is attributed to nonspecific aggregation. We were unable to fit the concentration distributions to a hetero-associative model, suggesting a very strong GABP<sub>a</sub>-GABP<sub>b</sub> interaction. Based on the instrument detection limit, the K<sub>e</sub> for the GABP αβ heterodimer was estimated to be below 10<sup>−8</sup> m<sup>−1</sup>. This estimated K<sub>e</sub> for the GABP αβ heterodimer is consistent with the reported K<sub>e</sub> (7.8 ± 0.63 × 10<sup>−10</sup> m<sup>−1</sup>) as measured by surface plasmon resonance (30). These results demonstrate that GABP under the experimental conditions and concentration range used in these experiments, exists exclusively as a stable heterodimer. Attempts to fit the data to a “stable heterotetramer” model (Fig. 4B, left panel) or to a two-species associative model (Fig. 4B, right panel) were unsuccessful.

**Deletion of the NH<sub>2</sub>-terminal Portion of GABP<sub>a</sub> Promotes Heterotetramer Formation in Solution**—Although our data indicate that GABPβ weakly dimerizes in solution, the GABP<sub>a</sub>β heterodimer exhibits no tendency to associate, even at concentrations above 2.5 μM. To resolve this apparent contradiction, we studied the association of GABP<sub>a</sub>Q and GABPβQ in solution. In EMSA experiments (Fig. 1), GABP<sub>a</sub>Q and GABPβQ predominantly form heterotetrameric complex when incubated with probe containing two PEA3/EBSs and displays a limited tendency to bind as a heterotetramer to DNA containing a single PEA3/EBS. To determine the associative state of GABP<sub>a</sub>Q and GABPβQ when free in solution, a slight excess of GABP<sub>a</sub>Q protein was combined with GABPβQ protein and centrifuged to equilibrium. The solute distribution was satisfactorily described by a heterodimer-heterotetramer equilibrium plus a third nonassociating species (Fig. 5, Equation 4). The molecular mass of the heterodimer was fixed at 61,525 Da, equal to the molecular mass of the His<sub>6</sub>-tagged GABPα<sub>cQ</sub>-GABPβ complex.

The molecular mass of the third species was fixed at 18,551 Da, equal to that of the His<sub>6</sub>-tagged GABPα<sub>cQ</sub> monomer, which was present in excess. Least-squares minimization yielded a dissociation constant for GABPα<sub>cQ</sub>-GABPβQ dimer-tetramer equilibrium equal to 0.17 ± 0.045 μM, significantly lower than that obtained for GABPβQ alone. These results suggest: 1) the COOH-terminus of GABP<sub>a</sub>Q promotes GABPβQ dimerization and 2) that the NH<sub>2</sub>-terminus of GABPβQ may interfere with heterotetramer complex assembly in solution. The latter conclusion explains, in part, the failure of the full-length GABP<sub>a</sub> protein to form heterotetrameric complex with GABP<sub>b</sub> protein in solution or bound to DNA containing a single PEA3/EBS.

**GABP Heterotetramer Complex Assembly Requires DNA Binding**

When stoichiometric amounts of GABP<sub>a</sub>Q, GABPβQ, and GABPβ proteins, containing a slight excess of the GABP<sub>a</sub>Q protein, was performed in a Beckman Model XL-1 ultracentrifuge at 20 °C, at speeds of 12,000, 16,000, and 20,000 rpm, and at initial protein concentrations between 0.2 and 0.7 A<sub>280</sub> units. Concentration distributions were fitted to a two-species associative model corrected for the presence of an excess of GABP<sub>a</sub>Q protein (Equation 4, under “Experimental Procedures”). The molecular weight of the monomer was equal to the sum of the molecular mass of the His<sub>6</sub>-tagged GABPα<sub>cQ</sub> and GABPβQ proteins. The molecular weight of the third species was equal to that of the His<sub>6</sub>-tagged GABPαQ protein.
various combinations (27, 32, 33). Our observation that GABP is of purified GABP solution, we have performed sedimentation equilibrium analysis to that of the His6-tagged GABP protein. Concentration distributions were fitted to an ideal single-species model (Equation 1, “Experimental Procedures”) with a molecular weight equal to the sum of the molecular weights of the His6-tagged GABP and GABP334 proteins. B, concentration distributions of a mixture of the GABPα and GABPβ334 proteins containing slight excess of the GABPβ334 protein, were fitted to an ideal two-species model (Equation 2, “Experimental Procedures”), with the molecular weight of one species equal to the sum of the molecular weights of the His6-tagged GABPα and GABPβ334 proteins, and the molecular weight of the other species equal to that of the His6-tagged GABPβ334 protein.

FIG. 6. Deletion of the COOH-terminal leucine zipper-like domain in GABPβ abolishes heterotetramer formation with GABPα,Q in solution. A, equimolar mixtures of GABPα,Q and GABPβ334 were centrifuged in a Beckman Model XL-I ultracentrifuge at 20 °C, at speeds of 12,000, 16,000, and 20,000 rpm, at initial protein concentrations between 0.4 and 1.3 M. Concentration distributions were fitted to an ideal single-species model (Equation 1, “Experimental Procedures”) with a molecular weight equal to the sum of the molecular weights of the His6-tagged GABPα,Q and GABPβ334 proteins. B, concentration distributions of a mixture of the GABPα and GABPβ334 proteins containing slight excess of the GABPβ334 protein, were fitted to an ideal two-species model (Equation 2, “Experimental Procedures”), with the molecular weight of one species equal to the sum of the molecular weights of the His6-tagged GABPα and GABPβ334 proteins, and the molecular weight of the other species equal to that of the His6-tagged GABPβ334 protein.

terminus leucine zipper-like domain of GABPβ is obligatory for formation of GABP heterotetramer complexes.

DISCUSSION

Since the discovery of GABP (1, 2, 18), it has been widely accepted that GABP exists in solution predominantly as a heterotetramer (αββα), due to stable β-β interactions (2, 3). This model was supported by subsequent observations that heterotetramer formation was required for full transcriptional transactivation by GABP (20, 21). Although attractive, this model is not consistent with the absence of heterotetramer complex in EMSA experiments performed with a DNA probe containing a single PEA3/EBS (Fig. 1). DNA containing two PEA3/EBSs is required to observe significant amounts of heterotetramer complex in EMSA experiments, although equivalent amounts of heterodimer complex is observed in these experiments. These results are inconsistent with a stable GABP heterotetramer complex binding as a single unit to DNA containing one or two PEA3/EBSs. Cellular proteins such as bcl3 can promote GABP heterotetramer formation in EMSA experiments (31), suggesting that the absence of the heterotetrameric complex does not merely reflect its instability under EMSA conditions, but that its formation may be a regulated process. Our observations suggest that the GABPαβ heterodimer is the major species found in solution and that efficient heterotetramer formation requires specific DNA for assembly.

To directly address the associative behavior of GABP in solution, we have performed sedimentation equilibrium analysis of purified GABPα, GABPβ, and several deletion mutants in various combinations (27, 32, 33). Our observation that GABPβ exists in a “monomer to dimer” equilibrium (Kd = 1.8 ± 0.4 μM), is incompatible with the “stable heterotetramer” model previously suggested to be required for heterotetramerization (1–3). Concentration distributions of an equimolar mixture of GABPα and GABPβ, however, best fit to a two-species model consisting of the GABPαβ heterodimer and a 619,573 Da species, which we attribute to the aforementioned nonspecific aggregation of GABPα. We have been unable to satisfactorily model the GABPαβ concentration distribution with an α + β ↔ αβ ↔ (αβ)2 equilibrium model. These results suggest (i) that GABP α and β subunits form a stable heterodimer in solution with an estimated Kd less than 10−8 M, and (ii) that, under our experimental conditions, no GABPαβ2 heterotetramer is observed in solution. Our estimation for a GABPαβ dissociation constant is consistent with the apparent Kd determined by Suzuki et al. (30) using surface plasmon resonance (Kd = 7.8 ± 0.63 × 10−10 M). Our results are also consistent with EMSA experiments suggesting that efficient heterotetramer formation in the absence of any exogenous regulatory influences occurs only on DNA with two or more PEA3/EBSs (Fig. 1).

GABPβ heterodimers assemble into the heterotetramer complex on DNA containing two PEA3/EBSs separated by as many as 4.0 helical turns (Fig. 2).2 The stability of the heterotetramer complex is greatly affected by the spacing between PEA3/EBSs such that, stability is enhanced on DNAs containing two PEA3/EBSs positioned on the same face of the DNA helix. These results are consistent with our previous data showing that GABP-dependent initiator activity is enhanced when two PEA3/EBSs are positioned on the same face of the DNA helix (23). Similarly, GABPβ isoforms or mutants lacking the COOH-terminal homodimerization domain are reportedly unable to promote heterotetramer complex formation and fail to activate transcription of linked promoters (20–22). The demonstration that heterotetramer complex stability correlates with GABP-dependent initiator activity further advances the notion that the heterotetramer complex is the functionally active form of GABP.

The NH2-terminal deletion mutant of GABPα (GABPα,Q) forms significant amounts of heterotetramer complex even on DNA with a single PEA3/EBS. This finding suggests that the NH2 terminus of GABPα may interfere with heterotetramer complex formation. In agreement with this observation, the concentration distribution of a GABPα,Q/GABPβ equilibrium mixture best fits a two-species associative model (Kd = 0.17 ± 0.045 μM) with the molecular weight of the monomeric species equal to that of the His6-tagged GABPα,Q/GABPβ dimer. Therefore, we have demonstrated that GABP exists as a stable heterodimer, and that multiple PEA3/EBSs are required to promote formation of the heterotetramer complex. Our observation that the NH2-terminal portion of GABPα subunit exerts an inhibitory effect on GABPαβ heterotetramer formation, and the observations of Shijo et al. (31) demonstrating the bcl3 enhances GABPαβ heterotetramer DNA binding, suggest that heterotetramer assembly is likely a regulated process that depends on specific promoter organization and/or specific regulatory proteins.
Homo- and heterodimerization is often used by DNA-binding proteins in order to increase specificity and affinity for a cognate binding site. Although preassembled multiprotein complexes may possess higher overall affinity, they also will have higher nonspecific affinity for DNA, and at limiting concentrations, a protein complex could become kinetically trapped at random positions in the genome (34, 35). Therefore, a high “off-rate” of a monomeric protein, compared with the fully assembled complex, will ensure that only high affinity sites will be occupied for a sufficient amount of time to enucleate the assembly of a higher order protein-DNA complex. This mechanism, designated as the “monomeric pathway,” has been proposed for the basic helix-loop-helix protein Max, the bZIP transcription factor ATF-2, and the Arc repressor (34, 36). Although preassembled multiprotein complexes may possess higher overall affinity, they also will have random positions in the genome (34, 35). Therefore, a high “off-rate” of a monomeric protein, compared with the fully assembled complex, will ensure that only high affinity sites will be occupied for a sufficient amount of time to enucleate the assembly of a higher order protein-DNA complex. This mechanism, designated as the “monomeric pathway,” has been proposed for the basic helix-loop-helix protein Max, the bZIP transcription factor ATF-2, and the Arc repressor (34, 36). Although preassembled multiprotein complexes may possess higher overall affinity, they also will have random positions in the genome (34, 35). Therefore, a high “off-rate” of a monomeric protein, compared with the fully assembled complex, will ensure that only high affinity sites will be occupied for a sufficient amount of time to enucleate the assembly of a higher order protein-DNA complex. This mechanism, designated as the “monomeric pathway,” has been proposed for the basic helix-loop-helix protein Max, the bZIP transcription factor ATF-2, and the Arc repressor (34, 36). Although preassembled multiprotein complexes may possess higher overall affinity, they also will have random positions in the genome (34, 35). Therefore, a high “off-rate” of a monomeric protein, compared with the fully assembled complex, will ensure that only high affinity sites will be occupied for a sufficient amount of time to enucleate the assembly of a higher order protein-DNA complex.

Acknowledgments—We thank Dr. Jeffrey Hansen for helpful comments and suggestions during the completion of these studies. We are grateful to Matt Stanley for help in preparing the manuscript and figures for publication.

REFERENCES
1. LaMarco, K., Thompson, C. C., Byers, B. P., Walton, E. M., and McKnight, S. L. (1991) Science 253, 789–792
2. Thompson, C. C., Brown, T. A., and McKnight, S. L. (1991) Science 253, 762–768
3. de la Brousse, F. C., Birkenmeier, E. H., King, D. S., Rowe, L. B., and McKnight, S. L. (1994) Genes Dev. 8, 1853–1865
4. Seth, A., Aucine, R., Fisher, R. J., Mavrothalassitis, G. J., Bhat, N. K., and Papas, T. S. (1992) Cell Growth Differ. 3, 327–334
5. Watanabe, H., Sawada, J., Yano, K., Yamaguchi, K., Goto, M., and Handa, H. (1993) Mol. Cell. Biol. 13, 1385–1391
6. Virbasius, J. V., and Scarpulla, R. C. (1993) Mol. Cell. Biol. 11, 5631–5638
7. Virbasius, J. V., and Scarpulla, R. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1309–1313
8. Virbasius, J. V., Virbasius, C. C., and Scarpulla, R. C. (1993) Genes Dev. 7, 380–392
9. Carter, R. S., and Avadhani, N. G. (1994) J. Biol. Chem. 269, 4381–4387
10. Carter, R. S., Bhat, N. K., Basu, A., and Avadhani, N. G. (1992) J. Biol. Chem. 267, 25418–25426
11. Baehman, N. J., Yang, T. L., Densen, J. S., Ernst, R. E., and Lonach, M. I. (1996) Arch. Biochem. Biophys. 333, 152–162
12. Villena, J. A., Vinas, O., Mampel, T., Iglesias, R., Giralt, M., and Villarroya, F. (1998) Biochem. J. 331, 121–127
13. Bottiger, E. P., Shelley, C. S., Farokhzad, O. C., and Arnaout, M. A. (1994) Mol. Cell. Biol. 14, 2604–2615
14. Rosmarin, A. G., Luo, M., Caprio, D. G., Shang, J., and Simkевич C. P. (1998) J. Biol. Chem. 273, 13097–13103
15. Hoffmeyer, A., Avost, A., Flory, A., Weber, C. K., Serfling, E., and Rapp, U. R. (1998) J. Biol. Chem. 273, 10112–10119
16. Banertz, N., Avost, A., Bæger, M., Serfling, E., and Kurth, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1541–1546
17. Markiewicz, S., Bosseult, R., De Leist, F., De Villartay, J-P., Hivroz, C., Ghyselael, J., Fisher, A., and De Saint Basile, G. (1996) J. Biol. Chem. 271, 14849–14855
18. LaMarco, K. L., and McKnight, S. L. (1989) Genes Dev. 3, 1372–1382
19. Martin, M. E., Piette, J., Yaniv, M., Tang, W-J., and Folk, W. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5839–5843
20. Sawada, J., Goto, M., Sawa, C., Watanabe, H., and Handa, H. (1994) EMBO J. 13, 1396–1402
21. Gugneja, S., Virbasius, J. V., and Scarpulla, R. C. (1995) Mol. Cell. Biol. 15, 102–111
22. Sawa, C., Goto, M., Suzuki, F., Watanabe, H., Sawada, J.-i., and Handa, H. (1996) Nucleic Acids Res. 24, 4954–4961
23. Yu, M., Yang, X-Y., Schmidt, T., Chinenov, Y., and Martin, M. E. (1997) J. Biol. Chem. 272, 29060–29067
24. Sucharov, C., Basu, A., Carter, R. S., and Avadhani, N. G. (1995) Gene Exp. 3, 93–111
25. Yoo, W., Martin, M. E., and Folk, W. R. (1991) J. Virol. 65, 5391–5400
26. Martin, M. E., Chinenov, Y., Yu, M., Schmidt, T. K., and Yang, X.-Y. (1996) J. Biol. Chem. 271, 25617–25623
27. Chinenov, Y., Schmidt, T., Yang, X-Y., and Martin, M. E. (1998) J. Biol. Chem. 273, 6203–6209
28. Laue, T. M., and Stanford, W. F., III (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 75–100
29. Brown, T. A., and McKnight, S. L. (1992) Genes Dev. 6, 2502–2512
30. Suzuki, F., Goto, M., Sawa, C., Ho, S., Watanabe, H., Sawada, J., and Handa, H. (1998) J. Biol. Chem. 273, 29392–29308
31. Shiio, Y., Sawada, J.-i., Handa, H., Yamamoto, T., and Inoue, J. (1996) Oncogene 12, 1837–1845
32. Hansen, J. C., Lebowitz, J., and Demeler, B. (1994) Biochemistry 33, 13155–13163
33. Minton, A. P. (1990) Annu. Rev. Biochem. 60, 1–6
34. Kühler, J. J., Metzlo, S. J., Schneider, T. L., and Schepartz, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11735–11739
35. Pomerantz, J. L., Wolfe, S. A., and Pabo, C. O. (1998) Biochemistry 37, 965–970
36. Reineiener, D., Johnson, T., and Sauer, R. T. (1999) Nat. Struct. Biol. 6, 569–573
The α and β Subunits of the GA-binding Protein Form a Stable Heterodimer in Solution: REVISED MODEL OF HETEROTETRAMERIC COMPLEX ASSEMBLY

Yurii Chinenov, Michael Henzl and Mark E. Martin

J. Biol. Chem. 2000, 275:7749-7756.
doi: 10.1074/jbc.275.11.7749

Access the most updated version of this article at http://www.jbc.org/content/275/11/7749

Alerts:

- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 25 of which can be accessed free at http://www.jbc.org/content/275/11/7749.full.html#ref-list-1