Coupling of Site-Specific DNA Binding to Protein Dimerization in Assembly of the Biotin Repressor−Biotin Operator Complex†

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ABSTRACT: The *Escherichia coli* repressor of biotin biosynthesis, BirA, binds site-specifically to the biotin operator, a 40 base pair imperfect inverted palindrome. Two repressor monomers have been shown to bind to the two operator half-sites. Analysis of results of quantitative DNase I footprint titrations performed on the wild-type biotin operator template indicate that binding is well described by a cooperative mechanism. The data obtained from these studies were, however, insufficient to independently resolve all of the energetic parameters associated with cooperative binding of the two repressor monomers to the operator site. In this work, to further dissect the energetics of assembly of the biotin repressor−biotin operator complex, measurements of binding of BirA to four bioO variants designed to reduce the valency of repressor binding from 2 to 1 have been performed. Results of these measurements indicate, as was found with the wild-type biotin operator template, that two repressor monomers bind simultaneously to the two half-sites of all variant operators. Protein dimerization and DNA binding are thus obligatorily coupled in the biotin repressor system. Furthermore, the results suggest that, in the context of a cooperative binding mechanism, the cooperative free energy associated with the biotin repressor−biotin operator interaction is significantly more favorable than the previously estimated −2 kcal/mol.

Coupling of protein assembly to site-specific binding of a regulatory protein to its target site on DNA is a common theme in formation of transcriptional regulatory complexes. In eukaryotes, a complex example of this phenomenon is the multiprotein preinitiation complex that forms at the transcriptional initiation regions of genes transcribed by RNA polymerase II (*1*). Even in simple systems in which a single protein binds to a target site, the contribution of a protein assembly process to the total stability of a transcriptional regulatory complex can be significant. To gain an understanding of the underlying physical chemistry of transcriptional regulation, both the mechanistic and equilibrium thermodynamic features of processes involving coupling of protein assembly to DNA binding must be elucidated. Moreover, the contribution of the assembly process to the observed cooperativity in many site-specific DNA binding reactions must also be ascertained.

Biotin biosynthesis in *Escherichia coli* is regulated at the transcriptional level (*2*). A schematic diagram of the biotin biosynthetic operon is shown in Figure 1A. The operon contains five of the six genes that encode biotin biosynthetic enzymes. The sixth biosynthetic gene, *bioH*, as well as the gene that codes for BirA, *bioR*, are located on separate regions of the *E. coli* chromosome. The repressor of biotin biosynthesis, BirA, binds to the 40 base pair biotin operator sequence (bioO) to prevent initiation of transcription from the divergent promoters, P_A and P_B, of the operon. BirA binds to bioO in a biotin-dependent mechanism in which the adenylated form of biotin, biotinyl-5′-AMP (bio-5′-AMP), functions as the positive effector for site-specific DNA binding (*5, 6*). No evidence supporting a role for BirA in

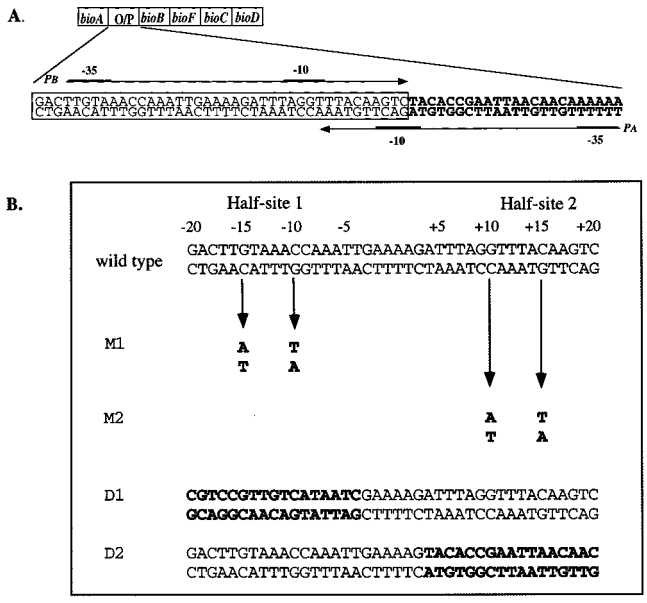

FIGURE 1: (A) Schematic representation of the *Escherichia coli* biotin operon. The five genes that code for biotin biosynthetic enzymes are *bioA*−*bioD* and *bioF*. The promoters that code for transcription initiation in the right and left directions, respectively, are P_B and P_A (*3, 4*). The boxed sequence in the expansion of the O/P region of the operon is that of the biotin operator. (B) Sequences of the wild-type biotin operator (*3*) and the mutant operator sites constructed for this work. The letters in boldface type indicate the sequence changes that were made in each of the mutant bioO sites.

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regulation of transcription of either *bioR* or *bioH* exists. An unique feature of this regulatory system is that the transcriptional repressor is also the enzyme that catalyzes covalent linkage of biotin to the biotin acceptor protein subunit of the acetyl-CoA carboxylase (*7*, *8*). Moreover, in this system the corepressor in site-specific binding of BirA to bioO, bio-5′-AMP, also functions as the activated intermediate in the biotin transfer reaction (*6*). BirA catalyzes synthesis of bio-5′-AMP from the substrates biotin and ATP.

BirA is a 35.3 kDa monomer, the structure of which has been determined by X-ray crystallography (*9*). The protein monomer contains three domains; N-terminal, central, and C-terminal domains. The fold of the N-terminal or DNA binding domain of BirA places it in the winged helix−turn−helix class of site-specific DNA binding proteins. The central domain is involved in both catalysis and DNA binding since it contains the binding site for bio-5′-AMP. The function of the C-terminal domain has yet to be determined. The biotin operator (bioO) sequence is a nearly perfect inverted palindrome and thus can be considered to be composed of two half-sites, which we have arbitrarily identified as half-sites 1 and 2 (Figure 1B). The adenylate-bound biotin repressor (holoBirA) has previously been shown to be monomeric at the concentrations at which it binds to the biotin operator sequence. The mechanism of site-specific DNA binding of holoBirA to bioO was reported to involve cooperative association of two holoBirA monomers to the two operator half-sites (*10*). The binding model is based on analysis of quantitative DNase I footprint titrations performed on a DNA template containing the wild-type bioO sequence. This limited footprint titration data is, however, insufficient for simultaneous resolution of the three parameters, the two intrinsic, ΔG_1 and ΔG_2, and the cooperative, ΔG_{12}, free energies that govern the holoBirA−bioO binding interaction (*11*). Rather, the intrinsic free energy for binding of holoBirA to each half-site was obtained from nonlinear least-squares analysis of the wild-type DNase I footprint titration data using fixed values for the cooperative free energy term. Further dissection of the physical chemical properties of the holoBirA−bioO interaction requires resolution of all three energetic terms that contribute to the binding process. This can be accomplished by measuring binding of holoBirA to reduced-valency biotin operator templates in which binding to one or the other half-site is eliminated. Results of measurements of binding to the reduced-valency templates should allow independent determination of the intrinsic Gibbs free energies for binding of the holoBirA monomer to each of the biotin operator half-sites. Simultaneous analysis of data obtained from a combination of wild-type and mutant templates will allow resolution of all three energetic terms governing the binding interaction.

To accomplish the objectives described above, four mutants of the biotin operator, each containing sequence alterations in a single half-site of the operator sequence, have been constructed. Results of DNase I footprint titrations performed on the altered templates indicate that holoBirA binds with considerably reduced affinity to each mutant relative to its binding to the wild-type bioO template. However, contrary to expectation, the mutant and wild-type half-operator sequences in all altered operator templates display similar dependencies of fractional occupancy on holoBirA monomer concentration. The data, moreover, provide no evidence for the existence of a singly liganded species in which one holoBirA monomer is bound to the wild-type bioO half-site at any repressor concentration. Results of nonlinear least-squares analysis of the titration data using a cooperative binding model indicate that holoBirA binds with the same cooperativity to the wild-type and altered bioO templates. The data have also been analyzed using an alternative binding model involving association of a preformed holoBirA dimer with the DNA. Comparison of results of analysis of the data obtained using the two models indicates that they cannot be distinguished with respect to the accuracy with which they describe the binding data. These results illustrate a fundamental difficulty associated with elucidating the mechanism of site-specific DNA binding of proteins that undergo weak oligomerization reactions.

MATERIALS AND METHODS

Chemicals and Biochemicals. All chemicals used in preparation of buffers were at least reagent grade. The α-^{32}P-labeled deoxyribonucleotides (3000 Ci/mmol) were purchased from Amersham. Unlabeled deoxyribonucleotide triphosphates were from P-L Biochemicals. Klenow fragment and restriction endonucleases were obtained from Promega, Inc. Electrophoresis-grade acrylamide and urea, both of which were manufactured by Eastman Kodak Co., were purchased from VWR. Bisacrylamide was obtained from Bio-Rad. All other chemicals were reagent or analytical grade. Acrylamide, bisacrylamide, and urea were deionized using the Bio-Rad AG501 mixed-bed resin prior to use. The biotin repressor, BirA, was purified as described in ref *10*. Protein concentration was determined by UV absorbance using an extinction coefficient at 280 nm of 1.3 mg^{-1} mL cm^{-1}.

Construction of Mutant Biotin Operator Templates. All mutant templates are derivatives of the plasmid pBioZ, the construction of which is described in ref *10*. The plasmid was constructed by inserting a biotin operator fragment obtained by restriction digestion of the plasmid pAOC3 (Matsuzaki and Otsuka, personal communication) with *Eco*RI into the *Eco*RI site of the vector pZ150 (*12*). Since the vector encodes both a ColE1 and an M13 origin of replication, the resulting plasmid can be propagated in either double- or single-stranded forms. The availability of the single-stranded form of the plasmid facilitates site-directed mutagenesis of the biotin operator sequence. Single-site and deletion mutants of bioO were constructed according to the oligonucleotide site-directed mutagenesis method described in ref 13 using a kit obtained from Amersham. The *E. coli* strain TG1 was transformed with the mutagenized plasmid, and selection for transformation was achieved by growth of the bacteria on LB medium containing 100 μg/mL ampicillin. Transformants were screened by restriction digestion of plasmids isolated from selected clones, and sequences were verified using the dideoxy chain-termination method (*14*).

Plasmids were purified from *E. coli* strain X90 using the method of Birnboim and Doly (*15*). All plasmids were subjected to a final purification step by cesium chloride density centrifugation and were stored at −70 °C in buffer containing 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA. The concentration of plasmid DNA was determined by UV absorption using an extinction coefficient at 260 nm of 50 μg^{-1} mL cm^{-1}.

Preparation of Fragments for DNase I Footprinting. Plasmids containing wild-type and mutant biotin operator fragments were first cleaved with the enzyme *Hin*dIII. The resulting product was end-labeled with ^{32}P using the Klenow fill-in procedure and the labeled DNA was separated from unincorporated nucleotides by anion-exchange chromatography on NACS Prepac Columns (Life Technologies, Inc.). The labeled DNA was cleaved with *Pst*I and resulting products were separated by agarose gel electrophoresis. After autoradiography, gel slices containing the operator fragments were excised and the DNA was purified by electroelution (*16*) followed by chromatography on a NACS Prepac column. Labeled fragments were suspended at a final concentration of <20000 dpm/μL in 10 mM Tris-HCl pH 8.0, and 0.1 mM EDTA and stored at 4.0 °C. Fragments were typically useful for quantitative footprinting for 1.5 weeks.

DNase I Footprinting. Qualitative and quantitative DNase I footprinting was performed using a modification of the method described by Brenowitz et al. (*11*). Binding reactions were prepared by combining labeled DNA (final concentration approximately 20 pM) with protein in buffer containing 10 mM Tris-HCl, pH 7.50 ± 0.01 at 20.0 ± 0.1 °C, 50 mM KCl, 1.0 mM $CaCl_2$, 2.5 mM $MgCl_2$, 100 μg/mL BSA, 20 μg/mL sonicated calf thymus DNA, 25 μM biotin, and 250 μM ATP and incubated at 20 °C for at least 1 h prior to DNase I cleavage. At these concentrations of biotin and ATP the biotin repressor is known to be saturated with bio-5′-AMP (*17, 18*). The DNase I was diluted just prior to use to a concentration of 0.2 μg/mL in buffer identical with that used for binding reactions minus BSA, calf thymus DNA, biotin, and ATP. Reactions were initiated by the addition of 5 μL of the DNase I solution to each 200 μL reaction mixture. After a 2 min incubation at 20 °C, the reaction was quenched by the addition of 33 μL of a 50 mM Na_2-EDTA solution. The DNA was precipitated by the addition of 700 μL of a solution containing 95% (v/v) absolute ethanol, 50 μg/mL yeast phenylalanyl-tRNA, and 0.31 M ammonium acetate. After centrifugation, the resulting pellets were washed twice with 800 μL of an 80% (v/v) absolute ethanol solution in water. Each DNA pellet was finally resuspended in 7 μL of a solution containing 80% (v/v) deionized formamide, 1× TBE, 0.02% (w/v) xylene cyanol, and 0.02% (w/v) bromophenol blue. Reaction products were separated by electrophoresis on 10% denaturing acrylamide gels and bands were visualized by autoradiography on X-OMAT-AR5 film (Kodak). The film was preflashed prior to autoradiography of the products of quantitative footprint titrations.

Chemical Sequencing of DNA. Chemical sequences of bioO templates were obtained on the identical fragments utilized for DNase I footprinting. Fragments were sequenced using the reactions specific for purine or pyrimidine bases as described by Maxam and Gilbert (*19*). The products of chemical cleavage were resolved parallel to the products of DNase I footprinting by electrophoresis on a 10% denaturing polyacrylamide gel.

Data Analysis. Autoradiographs of the results of quantitative DNase footprinting were digitized using the Molecular Dynamics personal laser densitometer. Corrections for deviation from linearity in the response of the instrument were made as described previously (*10*). Integrated optical densities for bands or blocks of bands in each operator half-site were obtained at each protein concentration and binding isotherms were generated as described in Brenowitz et al. (*11*). Binding data were analyzed by nonlinear least-squares techniques using NonLin (*20*). In all data analysis, the free holoBirA concentration is assumed to be equivalent to the total holoBirA concentration. The low concentration of DNA utilized in the binding reactions justifies this assumption. The binding models used in the analyses are discussed in the text.

RESULTS

Design of Mutant bioO Templates Utilized in DNase I Footprint Studies. The sequence of the biotin operator is shown in Figure 1. The operator sequence is a hyphenated inverted palindrome consisting of two 18 base pair segments interrupted by a 4 base pair A−T run (*3*). We have previously demonstrated that the mechanism of binding of holoBirA to the wild-type bioO sequence involves cooperative association of two protein monomers with the two operator half-sites (*10*). The cooperative free energy for the interaction is estimated to be between −2 and −3 kcal/mol in the buffer conditions employed in those experiments. The two intrinsic and the cooperative free energies governing the interaction could not, however, be unambiguously resolved using the data described in the previous work since only footprints of the wild-type bioO template were considered in the analysis. To resolve the three energetic terms related to cooperative binding of two holoBirA monomers to the two bioO half-sites, results of footprints obtained from reduced-valency operator templates in combination with the data obtained from footprints of the wild-type template are required (*11*). The changes introduced into the bioO sequence in order to create these "reduced-valency" templates for binding measurements are shown in Figure 1B. In each of the first two mutant templates, M1 and M2, two single base pair changes were introduced into one of the operator half-sites. The choice of the particular sequence changes was based on the results of combined genetic and biochemical studies of the holoBirA−bioO interaction. First, it has been demonstrated that the mutations introduced at positions −15 or +15 in the bioO sequence result in decreased affinity of holoBirA for the operator both in vivo and in vitro (*21*). Second, we have found that the guanine bases at positions −10 and +10 are altered in their susceptibility to chemical methylation when the operator is saturated with holoBirA (Streaker and Beckett, manuscript in preparation). In the second pair of "reduced-valency" templates, D1 and D2, either the first or second operator half-site, respectively, was deleted. Each 18 base pair operator half-site is, therefore, replaced by flanking sequences in these mutants. Note that the four central A−T base pairs are retained in both deletion mutants. All four mutant templates are expected to allow binding of only one holoBirA monomer to the wild-type half-site.

Sizes of the Footprints Obtained with Mutant Operator Templates. Results of qualitative DNase footprints obtained with the wild-type and mutant biotin operator templates are shown in Figure 2. Each of these footprints was obtained at saturating holoBirA concentration (see below). The end points of each 40 base pair operator sequence relative to each footprint are indicated in the figure. As demonstrated in the figure, the sizes of the footprints for all operator templates,

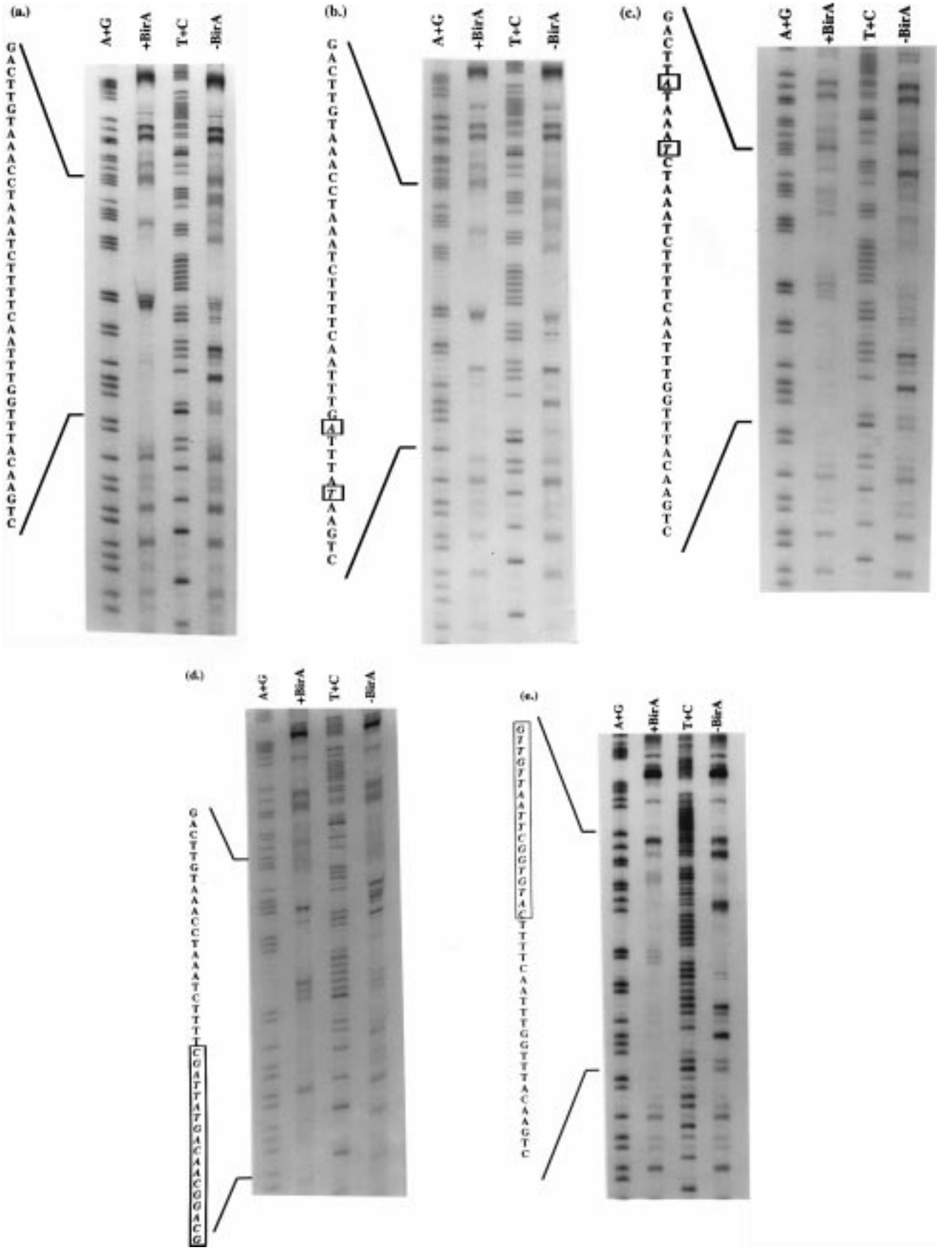

FIGURE 2: Qualitative DNase I footprints obtained with the wild-type and mutant bioO templates. Footprints were obtained as described in Materials and Methods. (A) Wild-type bioO; (b) bioO M1; (c) bioO M2; (d) bioO D1; (e) bioO D2. The holoBirA concentrations utilized for footprints of the wild-type and mutant templates are 5×10^{-7} and 1×10^{-6} M, respectively. The lanes labeled A + G and T + C refer to results of DNA sequencing of the templates performed using the chemical cleavage methods specific for purines or pyrimidines, respectively. The changes in the bioO sequence from the wild type are indicated by the boxed italicized letters.

including wild type and four mutants, are similar. We have previously demonstrated that the stoichiometry of the complex formed between holoBirA and wild-type bioO is 2 protein monomers/operator site (*10*). On the basis of the lengths of the protected regions observed with the mutant operators, we infer that the stoichiometries of the biotin repressor complexes formed with these templates are identical with that of the wild-type complex. Thus, contrary to expectation, both half-sites of all four mutant templates are functional in binding holoBirA.

Quantitative DNase I Footprints. To quantitate the interaction of holoBirA with each mutant template, equilibrium titrations were performed. A quantitative DNase I footprint titration of the bioO D1 template with holoBirA is shown in Figure 3. As indicated in the figure, the wild-type and nonspecific half-site in the template show very similar dependencies of protection from DNase I cleavage on holoBirA concentration. There is, moreover, no evidence for protection of fewer than 40 base pairs of sequence at any holoBirA concentration. Previously reported footprint titration data obtained with the wild-type bioO template were analyzed using the model shown in Figure 4A (*10*). In this model two protein monomers associate with the two operator half-sites. The two monomers can, in principle, associate either noncooperatively or cooperatively. Based on this model, the dependence of the fractional saturation of each operator half-site on holoBirA monomer concentration is given in

$$\bar{Y}_1 = \frac{k_1[\mathrm{P}] + k_1k_2k_{12}[\mathrm{P}]^2}{1 + (k_1 + k_2)[\mathrm{P}] + k_1k_2k_{12}[\mathrm{P}]^2} \quad (1)$$

$$\bar{Y}_2 = \frac{k_2[\mathrm{P}] + k_1k_2k_{12}[\mathrm{P}]^2}{1 + (k_1 + k_2)[\mathrm{P}] + k_1k_2k_{12}[\mathrm{P}]^2} \quad (2)$$

where $\bar{Y}_1$ and $\bar{Y}_2$ represent the fractional saturations of half-sites 1 and 2, respectively, by holoBirA monomer, [P] is the holoBirA concentration in monomer units, k_1 and k_2 are the intrinsic equilibrium constants governing binding of a monomer to half-sites 1 and 2, respectively, and k_{12} is the equilibrium constant associated with the cooperative interaction between the two monomers. In a noncooperative system, the value of the cooperativity parameter is 1. Results of analysis of the dependence of fractional saturation on protein concentration for binding of holoBirA to the wild-type and bioO D1 templates are shown in Figure 5. As indicated in the figure, the two half-sites of both the wild-type and mutant bioO templates exhibit identical dependencies of fractional saturation on holoBirA concentration. Significantly higher holoBirA concentrations are, however, required for binding of the mutant than for binding of the wild-type template. Titration data obtained with both the wild-type and D1 operator templates were analyzed using cooperative and noncooperative models for binding of the two monomers. The analyses were performed by fixing the cooperative free energy at particular values and fitting for the two intrinsic Gibbs free energy terms. The results of the analyses, which are shown graphically in Figure 5, indicate that binding of holoBirA to either the wild-type or bioO D1 template is better described by a cooperative model. Quantitative DNase I footprint titrations obtained with the three additional mutant operator templates, M1, M2, and D2, are similar to those obtained with the D1 template (data not shown). Most significantly, no evidence for the existence of a singly liganded species in which a holoBirA monomer is bound to only the wild-type operator half-site was present in titrations of these three mutant templates. The results of nonlinear least-squares analysis of footprint titration data obtained with all five templates using cooperative and noncooperative binding models are shown in Table 1. The footprint titrations described in this work were performed in buffer containing 50 mM KCl. As indicated in Table 1, the estimated upper limit on the cooperative free energy for binding of holoBirA to the wild-type biotin operator template in this buffer is approximately −2 kcal/mol, a value similar to that previously obtained in buffer containing 200 mM KCl (*10*). Any further decrease in the magnitude of the cooperative free energy term did not yield a smaller value for the square root of the variance of the fit and did lead to larger confidence intervals for the intrinsic Gibbs free energy terms (data not shown). The intrinsic Gibbs free energy for binding of holoBirA to each wild-type half-site is, however, more favorable by 0.5−1.0 kcal/mol at the lower salt concentration. Binding of holoBirA to each mutant template, as with the wild-type biotin operator template, is, as judged by the values of the square root of the variance of the fit, better described by a cooperative than a noncooperative model. An upper limit in the cooperative free energy of −2 kcal/mol is approximately the same for the mutant and wild-type templates. No improvement in the quality of the fits resulted from performing the analysis at more negative fixed values of the cooperative free energy term (data not shown). For all mutant templates the total Gibbs free energy for binding of holoBirA is significantly less favorable than that measured for the wild-type bioO template.

An alternative model for binding of holoBirA to the mutant operator templates involves association of a preformed holoBirA dimer with the template (Figure 4B). We have found from results of equilibrium analytical ultracentrifugation measurements that holoBirA undergoes limited assembly to dimer and that the equilibrium dissociation constant for this process is in the 10−100 μM range of holoBirA monomer concentration (Eisenstein and Beckett, manuscript in preparation). Given this equilibrium dissociation constant and the relatively weak affinity of holoBirA for the mutant templates, it is conceivable that the dimeric holoBirA species is present in sufficiently high concentration to play a significant role in binding to the mutant templates. This is unlikely for binding of holoBirA to the wild-type template since it occurs at significantly lower protein concentrations than the range relevant to the monomer−dimer equilibrium process. The dependence of fractional saturation of the operator site on holoBirA monomer concentration for a model involving association of a preformed dimer with the site is given in

$$\bar{Y} = \frac{[\mathrm{P}]^2}{KK_\mathrm{D} + [\mathrm{P}]^2} \quad (3)$$

where $\bar{Y}$ represents the fractional saturation of the entire operator site, [P] is the holoBirA monomer concentration, K_D is the equilibrium dissociation constant governing the dimerization process, and K is the equilibrium dissociation

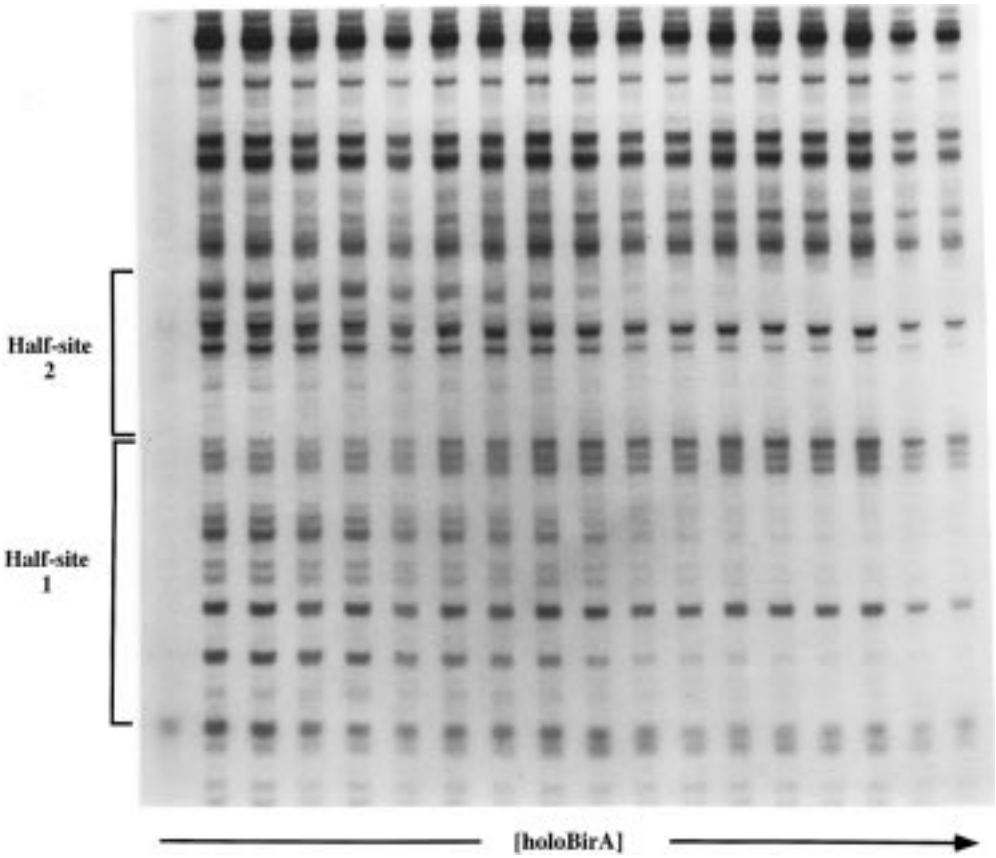

FIGURE 3: Results of quantitative DNase I footprinting of the bioO D1 template with holoBirA. The footprint was performed as described in Materials and Methods. The half-sites 1 and 2 are as indicated in Figure 1 and the protein concentrations are indicated in Figure 5.

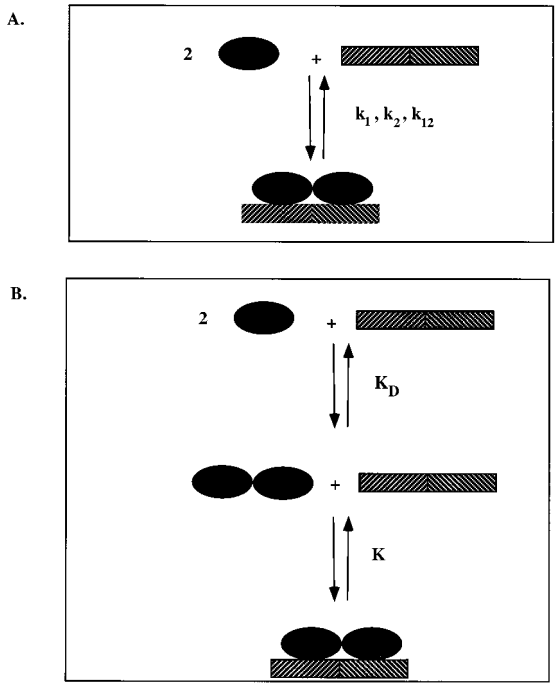

FIGURE 4: Schematic representation of the two alternative models for binding of holoBirA to an operator template. (A) Binding of two BirA monomers to the two operator half-sites. (B) Binding of a preformed holoBirA dimer to the template. The equations relating fractional saturation of the template to holoBirA concentration are presented in Results.

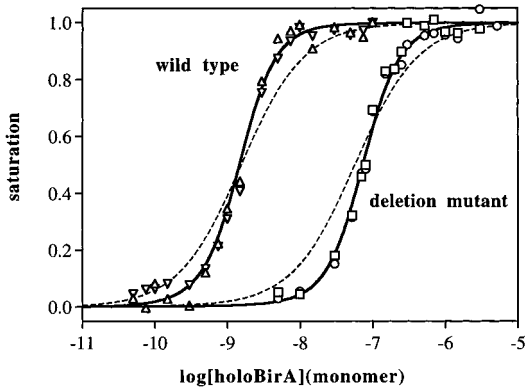

FIGURE 5: Individual site binding curves for holoBirA binding to the two half-sites of wild-type and D1 bioO templates. Wild-type: half-site 1 (△), Half-site 2 (▽). D1: half-site 1 (□), half-site 2 (○). The sequences of the half-sites of each template are indicated in Figure 1. The solid and dashed lines shown with each data set represent the best-fit individual site binding curves for half-site 1 obtained from nonlinear least-squares analysis of the data assuming cooperative free energies, ΔG_{12}, equal to −2 and 0 kcal/mol, respectively.

constant governing binding of the preformed dimer to the operator site. Results of analysis of the footprint titration data obtained for the D1 template shown in Figure 6 indicate that the data are well described by this alternative model. Results of application of this model to analysis of footprint titration data obtained with all of the mutant templates are shown in Table 2. A comparison of the goodness of fit for the analyses of the data using the models for cooperative association of two monomers with the templates and association of a preformed dimer with the template indicate that, with the exception of the D2 template, the quality of the fits is indistinguishable. Consequently, we are unable to determine which of the two models better

Table 1: Results of Analysis of Quantitative DNase Footprint Titration Data Obtained from Wild-Type and Mutant BioO Templates: Binding of Two HoloBirA Monomers[a]

BioO template[b]	ΔG_{12}[c] (kcal/mol)	ΔG_1 (kcal/mol)	ΔG_2 (kcal/mol)	σ[d]
wild type	0	-11.8 ± 0.3	-11.7 ± 0.3	0.078
	-1	-11.4 ± 0.2	-11.2 ± 0.2	0.049
	-2	-11.0 ± 0.3	-10.6 ± 0.3	0.036
M1	0	-9.4 ± 0.2	-9.3 ± 0.3	0.061
	-2	-8.6 ± 0.3	-8.2 ± 0.3	0.036
M2	0	-9.5 ± 0.2	-9.6 ± 0.3	0.049
	-2	-8.4 ± 0.4	-8.6 ± 0.4	0.032
D1	0	-9.7 ± 0.3	-9.7 ± 0.3	0.065
	-2	-8.6 ± 0.3	-8.5 ± 0.3	0.035
D2	0	-9.3 ± 0.3	-8.9 ± 0.3	0.071
	-2	-8.8 ± 0.3	-7.5 ± 0.3	0.037

[a] Data were obtained in buffer containing 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, and 1.0 mM $CaCl_2$, pH 7.50 ± 0.02 at 20.0 ± 0.1 °C, with 20 μg/mL calf thymus DNA and 100 μg/mL bovine serum albumin. The model used in the analysis is shown in Figure 4a. [b] Sequences of the indicated templates are shown in Figure 4A. [c] ΔG_{12} was fixed at the indicated values in the analysis. [d] Square root of the variance of the fit.

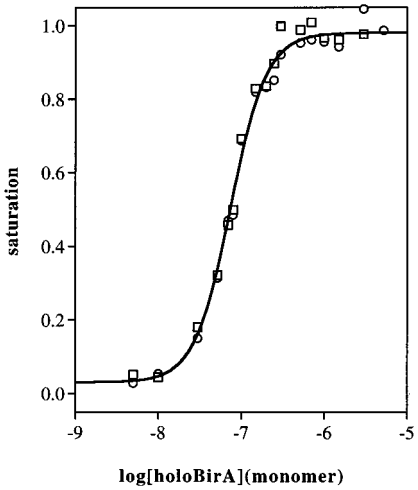

FIGURE 6: Results of nonlinear least-squares analysis of the data obtained from quantitative DNase I footprint titration of the D1 template using a binding model involving association of the preformed dimer with the template. The significance of the symbols is identical with that indicated in Figure 5.

Table 2: Results of Analysis of Quantitative DNase Footprints of Mutant bioO Templates: Binding of a Preformed Dimer to the Template[a]

BioO template	K_D[b] (M)	ΔG (kcal/mol)	σ[c]
M1	2×10^{-5}	-12.5 ± 0.1	0.039
M2	2×10^{-5}	-12.8 ± 0.1	0.048
D1	2×10^{-5}	-12.8 ± 0.1	0.035
D2	2×10^{-5}	-11.9 ± 0.1	0.074

[a] Buffer is identical to that indicated in Table 1. The data were analyzed using the model shown in Figure 4B. [b] The value of the equilibrium dissociation constant for dimerization of holoBirA was fixed at that measured by equilibrium analytical ultracentrifugation. [c] Square root of the variance of the fit.

describes the data for the three mutant bioO templates D1, M1, and M2.

Based on the resolved parameters obtained from nonlinear least-squares analysis of the DNase I footprint titration data using either binding model, the total Gibbs free energy for binding of holoBirA to each mutant template is 4−5 kcal/mol less favorable than it is for binding of the repressor to the wild-type template. This magnitude of the $\Delta\Delta G_{tot}$ is similar for all of the mutant templates examined, regardless of the nature of the change in the operator sequence.

DISCUSSION

Binding of holoBirA to bioO has previously been shown to involve cooperative association of two protein monomers with the two operator half-sites (*10*). The original intent of measuring binding of holoBirA to the mutant operator templates described in this work was to allow resolution of the three energetic terms, ΔG_1, ΔG_2, and ΔG_{12}, describing cooperative association of the two holoBirA monomers to the two biotin operator half-sites. The footprint titration data obtained with the mutants constructed for this work clearly indicate that none of them is a true "reduced-valency" template. First, the qualitative footprints of the mutant templates obtained at saturating holoBirA concentration are identical in size to the footprint obtained with the wild-type biotin operator template. Second, in quantitative footprints obtained with the mutant templates, no evidence exists at any repressor concentration to support the contribution of a singly liganded operator species, in which one holoBirA monomer is bound to the wild-type operator half-site, to the species population. All occupied biotin operator templates thus have two holoBirA monomers bound to them.

Lin and Shiuan (*22*) have described qualitative DNase I footprints of single-site and deletion mutants of bioO with holoBirA. These footprints were performed at a single holoBirA concentration at which the operator was fully occupied. Consistent with the footprints of the double mutant M1 and M2 templates reported in this work, the footprints obtained with single-site mutants at positions −15 or +15 in the sequence were identical in length to that obtained with the wild-type template. However, these researchers also reported footprints of a half-site deletion mutant of bioO that contained residues +2 to +33 of the operator sequence (see Figure 1 for numbering scheme). The size of the footprints obtained with this deletion mutant were only 12−13 base pairs in length. Furthermore, these researchers independently measured the stoichiometry of the complex formed between holoBirA and the deletion mutant and found it to contain 2 holoBirA monomers/site. They conclude that the monomer that interacts with the nonspecific region of the template is in the vicinity but not in direct contact with the DNA. The two deletion mutants described in this work, D1 and D2, were constructed by deleting base pairs −3 to −20 or +3 to +20, respectively from the operator sequence and replacing them with sequences that normally flank the operator sequence in the biotin operon. For both of these mutants the size of the footprint obtained with holoBirA is approximately 40 base pairs. The size of the footprint is, moreover, independent of protein concentration over the entire range of the titration curve. In both deletion mutants described in this work the $(A-T)_4$ sequence present in the wild-type operator has been retained. This is not true for the deletion mutant described by Lin and Shiuan. Perhaps this sequence, which constitutes a site of enhanced cleavage by DNase I in the complexes formed between wild-type or mutant operators with holoBirA (see Figure 2), is significant for the nature of the contacts formed between one of the repressor monomers and the noncognate half-site in the

complex. Alternatively, flanking sequences +21 to +33 that are present in the mutant templates described in this work but are absent in the +2 to +33 deletion mutant of Lin and Shiuan may be significant in formation of the holoBirA−bioO complex.

HoloBirA binding to the four mutant templates is characterized by a significantly lower stability than is its binding to the wild-type biotin operator. The destabilization in terms of the total Gibbs free energy for binding to the mutant templates as compared to the wild-type bioO template ranges from 4.1 kcal/mol for D1 to 5.3 kcal/mol for the D2 template. Interestingly, the destabilization of the holoBirA−operator complex caused by introduction of mutations into a single bioO half-site measured in this work is similar in magnitude to estimates based on in vivo measurements of transcriptional repression (*4*). Binding of holoBirA to either class of mutant template is specific, as demonstrated by the specificity of the footprints obtained with the templates as well as the fact that nonspecific binding in this system appears to be very weak. No nonspecific component to the binding of holoBirA to DNA has been detected, even in buffers containing KCl at the relatively low concentration of 50 mM (Beckett, unpublished results). The destabilization of the holoBirA−bioO interaction resulting from introduction of two point mutations into either half-site sequence is, thus, similar to that resulting from replacement of either specific half-site sequence with nonspecific flanking sequence.

The quantitative DNase I footprinting data obtained with the four mutant operator templates described in this work have been analyzed using two binding models. One model involves cooperative association of two holoBirA monomers with the two operator half-sites. Since both half-sites of the mutant templates, like those in the wild-type template, show identical dependencies of fractional occupancy on holoBirA monomer concentration, analysis of the DNase I footprinting data obtained with all five templates was performed by fixing the cooperative free energy for the interaction at a range of values and obtaining the best-fit values of the intrinsic free energies for binding of holoBirA to each operator half-site. As indicated in Table 1, within the limitations imposed by this analysis, holoBirA binds with similar cooperativity to the wild-type and mutant templates. Moreover, in agreement with previous results of DNase I footprints obtained at a higher salt concentration (*10*), the values of the two intrinsic free energies for binding of holoBirA to the wild-type template are identical. This is not unexpected since the operator site is an inverted palindrome in which the sequences of the two half-sites are nearly identical. However, results of analysis of the footprinting data obtained with the four mutant templates yield values of the two intrinsic free energies for binding to the mutant and wild-type half-sites that are also apparently similar in magnitude. It is unlikely that this result reflects the true affinities of a holoBirA monomer for two half-sites that differ significantly in sequence. Rather, it is an artifact of the manner in which the data were analyzed. Calculation of the species population distributions discussed below indicates that if association of the two holoBirA monomers does occur by a cooperative mechanism, then the cooperative free energy term associated with the binding process is considerably more favorable than the −2 kcal/mol that was previously estimated and utilized in the data analysis presented in this work.

Table 3: Microscopic Configurations and Associated Free Energies for the HoloBirA−bioO Binding Interaction[a]

species	operator configurations: bioO half-site 1	operator configurations: bioO half-site 2	free energy contribution ΔG_i
1	0	0	reference state
2	holoBirA	0	ΔG_1
3	0	holoBirA	ΔG_2
4	holoBirA ↔ holoBirA		$\Delta G_1 + \Delta G_2 + \Delta G_{12}$

[a] Biotin operator half-sites are denoted 0 if vacant or holoBirA if occupied by a holoBirA monomer. The double arrow connecting the two holoBirA monomers in species 4 indicates the cooperative interaction between the simultaneously bound monomers. ΔG_1 and ΔG_2 are the intrinsic free energies for interaction of the holoBirA monomer with half-sites 1 and 2, respectively. ΔG_{12} is the free energy of interaction of two holoBirA monomers bound simultaneously to bioO half-sites 1 and 2. The term ΔG_i is the sum of the contributions of the microscopic free energies for a given species.

If it is assumed that the model involving cooperative binding of two holoBirA monomers to the two bioO half-sites accurately describes the assembly of the complex, results of DNase I footprint titrations of the mutant operator templates can be utilized to obtain an upper limit for the cooperative free energy associated with the binding interaction. This is evident from calculations of the contribution of each operator species to the total species population in the titration experiments. The four species that contribute to binding of holoBirA to bioO in a cooperative binding model are shown in Table 3 along with the energetic contribution associated with each. The fractional contribution, f_i, of each species to the total species population at any holoBirA monomer concentration can be calculated from

$$f_i = \frac{\exp(-\Delta G_i/RT)[\text{holoBirA}]^j}{\sum_i \exp(-\Delta G_i/RT)[\text{holoBirA}]^j} \qquad (4)$$

where ΔG_i is the sum of the contributions of the microscopic free energies associated with a given species (i), [holoBirA] is the concentration of free holoBirA monomer, and j is the number of holoBirA monomers bound to a bioO operator template in the ith configuration. The fractional contributions of the individual species obtained for binding of holoBirA to the wild-type bioO template were calculated using the three energetic parameters obtained from analysis of the data based on a fixed value of the cooperativity parameter, ΔG_{12}, of −2 kcal/mol. Results of this calculation are shown in Figure 7A. Consistent with the titration data (see Figure 5), only two species corresponding to the unliganded, f_1, and the fully liganded, f_4, template, are predicted to be observable in titrations of the wild-type bioO template. A similar calculation can be performed on the basis of analysis of titrations of the mutant templates. As indicated from the data presented in Table 1, the total Gibbs free energy for binding of holoBirA to each mutant template is less favorable than that obtained for binding to the wild-type template by approximately 5 kcal/mol. In a cooperative system we assume that this $\Delta\Delta G_{tot}$ is only a consequence of the less favorable interaction of the holoBirA monomer with the altered half-site and that the intrinsic free energy for binding of the holoBirA monomer to the wild-type half-site, ΔG_1,

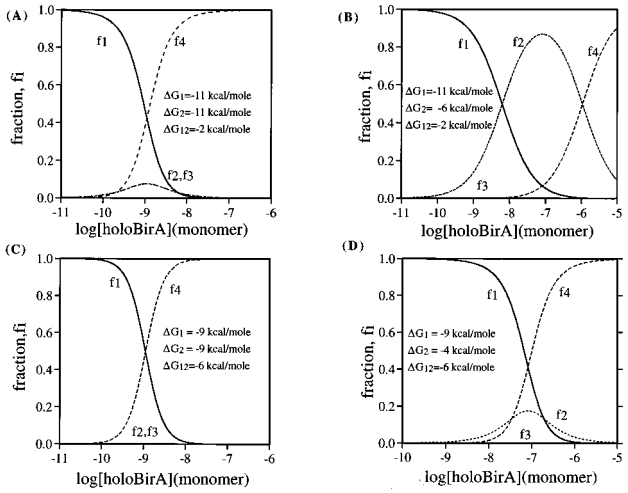

FIGURE 7: Population distributions of the species shown in Table 3 as a function of log [holoBirA] (monomer). (A) Wild-type bioO, $\Delta G_{12} = -2$ kcal/mol. (B) Mutant bioO, $\Delta G_{12} = -2$ kcal/mol. (C) Wild-type bioO, $\Delta G_{12} = -6$ kcal/mol. (D) Mutant bioO, $\Delta G_{12} = -6$ kcal/mol. The total Gibbs free energy for binding of holoBirA to wild-type bioO, ΔG_{TOT}, is -24 kcal/mol in both panels A and C. Binding of holoBirA to the mutant template, panels B and D, is characterized by a total Gibbs free energy of -19 kcal/mol.

as well as the cooperative free energy, ΔG_{12}, terms are unchanged from the values obtained in measurements of binding to wild-type bioO. Results of calculations of the species populations for the case in which half-site 2 is mutated are shown in Figure 7B. This figure indicates that if the magnitude of the cooperative free energy is -2 kcal/mol, species 2, the singly liganded template in which the wild-type half-site is occupied by a holoBirA monomer, should contribute significantly to the total species populations over a relatively broad range of holoBirA concentration. Moreover, the simulation predicts that the doubly liganded species should be present at significant levels only at relatively high holoBirA concentrations. The footprint titrations obtained with the mutants indicate that only two species, 1 and 4, contribute significantly to the total species population in the entire range of the titration curve. This experimental observation suggests that if binding occurs via a cooperative mechanism, the cooperative free energy in the system is significantly more favorable than -2 kcal/mol. This is illustrated in Figure 7C,D. In panel C the species populations are calculated assuming a total Gibbs free energy for binding identical with that determined for binding of holoBirA to the wild-type template, a cooperative free energy of -6 kcal/mol, and identical intrinsic free energies for binding of holoBirA to the two half-sites. Results of the calculations are consistent with the experimental observation of only two species in the titration curves. Interestingly, comparison of Figure 7 panels A and C illustrates the difficulty encountered in distinguishing highly cooperative from moderately cooperative binding to the wild-type template. The species populations for titration of a mutant template in which all of the loss in the energetics of binding reaction is associated with the intrinsic Gibbs free energy for binding of holoBirA to the mutant half-site 2, ΔG_2, are shown in Figure 7D. Consistent with the data obtained with the mutant templates, only two species contribute significantly to the total species population in this highly cooperative system. In the mutant, the simultaneous titration of the weak or altered half-site and strong or wild-type half-site can be understood in terms of the well-documented preferential partitioning of cooperative free energy to loading of a weak site in a two-site cooperative system (*23*). Additional simulations have been performed in which the cooperative free energy was assumed to be -3, -4, and -5 kcal/mol (data not shown). Agreement with the experimental results, however, requires an upper limit on the cooperative free energy of -6 kcal/mol.

A second model for binding used in analysis of the footprinting data involves association of a preformed holoBirA dimer with the operator site. Results of analysis of the DNase I footprint titrations of the mutant templates using this second model demonstrate that the goodness of fit is similar to that obtained using the model involving cooperative association. This conclusion is based on the similar values for the square root of the variance of the fit. Justification of application of the second model to analysis of the footprint titration data obtained with the mutant templates is based on the equilibrium constant for dimerization of holoBirA measured by equilibrium sedimentation (Eisenstein and Beckett, manuscript in preparation). The equilibrium dimerization constant measured in buffer of the same composition as that used for footprint titrations described in this work is 20 μM. Binding of holoBirA to the mutant templates occurs in the 0.1 μM range of holoBirA monomer concentration. In this concentration range the free dimer contributes less than 1% to the total holoBirA population. Even at this low level the dimer could be the preferred species in binding to the mutant templates.

In general, transcriptional regulatory proteins bind to their target sites on DNA in oligomeric forms. For systems in which the oligomerization of the free protein is energetically very favorable, as observed in studies of the bacteriophage λ cI repressor (*24*) and the trp repressor (*25*), binding occurs via association of a preformed oligomer to the target site. In cases in which the oligomerization process is either undetectable or very weak, it is uncertain if the protein assembly precedes or occurs concomitant with DNA binding. Systems in which this weak dimerization has been observed include the LexA repressor from *E. coli* (*26*, *27*) and the DNA binding domain (DBD) of the glucocorticoid receptor (*28*). On the basis of the size of footprints obtained with a half-operator construct of the RecA operator as well as comparisons of binding of the intact protein and LexA DNA binding domain to intact and half-operator sites, LexA is thought to bind to its dyad symmetric site via a monomer−DNA intermediate (*27*). A similar mechanism, based on detection of an intermediate complex as well as undetectably weak dimerization of the protein, has been proposed for binding of the glucocorticoid receptor DBD to the glucocorticoid response element. Results presented in this work indicate that for the biotin repressor system the validity of either of the two binding models cannot be established on the basis of results of equilibrium thermodynamic binding measurements alone.

The absence of evidence for the formation of any holoBirA−DNA complexes in which a single monomer is bound to a wild-type operator half-site has implications for the energetic basis of site-specific binding of this protein to DNA. The results indicate that dimerization of holoBirA is necessarily or obligatorily linked to its association with DNA.

As indicated from results of analysis of the quantitative DNase I footprinting data, the assembly may occur in the course of formation of the protein−DNA interface or prior to DNA binding. Elucidation of the precise mechanism of the assembly process will require application of kinetic methods (*29*). Regardless of the mechanistic details of the binding reaction, the protein−protein interaction contributes significantly to the overall stability of complexes formed between holoBirA and its target site on DNA.

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