RAR Antagonists Diminish the Level of DNA Binding by the RAR/RXR Heterodimer†

Nicolas Poujol, Emmanuel Margeat,‡ Stephanie Baud, Catherine A. Royer*

Centre de Biochimie Structurale, 29 rue de Navacelles, 34090 Montpellier CEDEX, France

Received October 23, 2002; Revised Manuscript Received January 22, 2003

ABSTRACT: A purified RAR/RXR-ΔAB heterodimer was obtained by production of His-tagged RAR and untagged RXR in *Escherichia coli*, followed by combined purification on a Ni^{2+} affinity column using excess RXR extract, and finally a gel filtration chromatography step to isolate a pure heterodimer. The purified heterodimer preparation bound 9-cisRA at a level of 0.85−0.95 mol of binding sites per mole of protein monomer. Titration of a 26 kDa fluorescent labeled fragment of the SRC-1 coactivator protein with the purified heterodimer in the presence of the agonist 9-cisRA yielded a binding affinity near 300 nM, whereas no binding was observed in the absence of agonist. Binding of the purified heterodimer to a DR5 target was identical in the absence of ligand and in the presence of 9-cisRA. Competition by unlabeled specific and nonspecific DNA allowed us to demonstrate that the binding curve was bimodal. The first phase of binding was highly specific and of high affinity. This phase also exhibited a high degree of cooperativity in the binding profile. Nonspecific DNA efficiently competes for the second phase. Thus, the first phase of binding likely corresponds to the formation of the specific heterodimer complex in which heterodimerization is energetically coupled to DNA binding. While agonist binding had no effect on the apparent affinity of the heterodimer for DR5, a series of antagonists significantly destabilized the heterodimer−DR5 complex, either through a direct decrease in the affinity of the protein for the DNA or through destabilization of the heterodimer itself. Impeding the interaction between the heterodimer and DNA appears as an additional mechanism of antagonist action of varying efficiency, depending upon the chemical structure of the antagonist.

The retinoic acid receptor (RAR) and the retinoic X receptor (RXR) are nuclear receptor proteins which, upon formation of RAR/RXR heterodimers, regulate gene expression in response to retinoid ligands (*1*). In particular, these proteins are implicated in signaling in vertebrate morphogenesis, differentiation, and homeostasis. While RXR can form heterodimers with a number of other nuclear receptors such as TR, VDR, and PPAR (*2*−*5*), it can also homodimerize (*4*) and even form homotetramers (*6*). Within the RAR/RXR heterodimer, the RAR partner confers the specific response to all-*trans*-retinoic acid, a response which can be potentiated by agonist binding to RXR (*7*).

The nuclear receptor proteins are modular in their structure and include an N-terminal transactivation domain (AB) which is not essential in the function of the RAR/RXR heterodimer (*8*), a DNA binding domain (DBD) that is highly conserved among all nuclear receptors, a linker domain showing low levels of homology among different nuclear receptors, and a ligand binding domain (LBD) that exhibits a ligand inducible transcriptional activation function (AF-2) in most receptors and that has evolved to accommodate the large variety of ligands recognized by these proteins. Transcriptional activation by the AF-2 involves ligand-mediated conformational changes in the LBD, resulting in either recruitment or dissociation of coregulator proteins. These coregulators are implicated in chromosome remodeling and interactions with the general transcription machinery (*9*, *10*). Like most of the other nuclear receptor proteins, both the DBD and the LBD of the RAR/RXR family of nuclear receptors present dimerization motifs (*11*−*17*), although the energetics of dimerization are much more favorable in the case of many of the LBDs, whereas the dimerization of the DBD is so weak it occurs only on DNA.

The formation of RXR-containing heterodimers determines the specificity of the DNA target site which, unlike the hormone receptor family proteins which recognize palindromes, consists of direct repeats of the target half sites (for a review, see refs *18* and *19*). Although the target half-site sequence is identical (AGGTCA) for all of the different RXR-containing heterodimers, the partner of RXR in the heterodimer determines the half-site spacing. Thus, RXR homodimers and RXR/RAR, RXR/PPAR, RXR/COUP, and RXR/HNF4 heterodimers recognize half-sites separated by one base pair (DR1), while RXR/VDR heterodimers recognize a three-base pair spacing (DR3). In the case of a RXR/LXR or RXR/TR heterodimer, the target site spacing is four base pairs (DR4), while the RXR/RAR heterodimer also

† This work was funded by the CNRS, INSERM, La Fondation pour la Recherche Médicale, L'Association pour la Recherche sur le Cancer, and the Région Languedoc-Roussillon. N.P. was a postdoctoral fellow (INSERM poste d'accueil recherche clinique), and E.M. was supported by a doctoral grant from the French Ministère de l'Education, de la Recherche et de la Technologie.

* To whom correspondence should be addressed. E-mail: royer@cbs.cnrs.fr.

‡ Current address: Department of Chemistry and Biochemistry, UCLA, Los Angeles, CA 90095.

10.1021/bi027056l CCC: $25.00
Published on Web 04/09/2003

binds specifically to a DR5 target (five-base pair spacing); binding to the DR5 is more favorable than to the DR1.

The structural consequences of such diversity in half-site spacing are quite intriguing, since they lead to both differences in the distance between the DBDs in the dimer and differences in their relative orientation (*13*−*16*). In addition to changes in the rotation of one subunit with respect to the other, in the case of the TR/RXR heterodimer, RXR occupies the 5′ position on the target DR4, while in the case of the RXR/RAR heterodimer, RXR binding is on the 3′ side. Thus, the protein−protein interactions of the DBDs in these different homo- or heterodimer−DNA complexes are significantly different. Although, as cited above, we now have considerable structural information concerning the interactions between the DBDs on targets with different spacings, little quantitative energetic data have been published on the coupling of DNA binding and heterodimerization between RXR and its partners. Moreover, since no structure of a full-length nuclear receptor is available, the structural and energetic consequences of differences in the heterodimeric structure of the DBDs bound to DNA on the dimerization of the LBDs have not been elucidated. Since ligand binding is known to affect the structure and dynamics of the LBDs of these proteins and, in the case of the RAR/RXR heterodimer, to engender allosteric effects in the heterodimer (*20*−*22*), the characterization of the effects of ligand binding on the heterodimer−DR5 interaction provides information about the energetic coupling between these interactions that are key to retinoid signaling.

Indeed, nuclear receptor function implicates a large number of coupled molecular interactions, including, at the very least, ligand binding, homo- and heterodimerization, protein−DNA binding, and coregulator fixation and competition. The specific function and, ultimately, the particular physiological role of the individual nuclear receptors are defined by the structure−function relations that govern these coupled interactions. It is therefore important to characterize their energetics in as much detail as possible. In the study presented here, we focus on the binding of the RAR/RXR-ΔAB heterodimer to a DR5 target and the effects of ligands, agonist and antagonist, on this interaction. Such studies are typically contingent upon obtaining reasonably large quantities of pure, active protein. In this case, we have prepared pure, functional RAR/RXR-ΔAB heterodimers, with residues deleted only in the N-terminal AB region. To define the properties specific to the proteins themselves, and thus to separate effects that could arise from particular cellular environments, we have chosen to perform high-sensitivity DNA binding assays *in vitro* using these purified RAR/RXR-ΔAB heterodimers. These assays are based on changes in the anisotropy of fluorescence of a labeled target oligonucleotide upon binding to the protein and are carried out in solution, thus ensuring true equilibrium conditions (*23*). The quality of the data and the sensitivity of the technique are such that both affinity and cooperativity can be obtained from the binding profiles.

MATERIALS AND METHODS

Copurification of a Homogeneous RAR/RXR Heterodimer. Our aim was to purify a RAR/RXR-ΔA/B heterodimer without any measurable contamination of the RXR homodimer which can bind with considerable efficiency to DR5 (*24*). The copurification strategy was similar to that described by Iyer and co-workers (*25*), except that it was carried out in the absence of a retinoid specific ligand and the DR5 oligonucleotide. Briefly, only RAR ΔA/B, which is always isolated as a monomer (*26*), was His-tagged (pET15bRARα ΔA/B, pET11RXRα ΔA/B). These plasmids express functional DNA and ligand-binding domains, but lack the N-terminal region. Once the extract of *E. coli* programmed to express His-RAR ΔA/B and RXR ΔA/B were mixed, the His-RAR/RXR heterodimer was formed. Only the His-RAR/RXR heterodimer and His-RAR were immobilized during the metal affinity chromatography, while excess RXR was not. To minimize the retention of monomeric RAR, we typically resuspended the pellets in 4 L RXR and 2 L RAR cultures. Fractions eluted from the Ni^{2+} affinity column were analyzed by SDS−polyacrylamide gel electrophoresis and Coomassie staining, pooled, and concentrated. To remove the putative excess of the His-RAR monomer, we performed a gel filtration (Superdex 75 column). Fractions were then analyzed by SDS−polyacrylamide gel electrophoresis and Coomassie staining, pooled, and concentrated to 20×10^{-6} M. The results of a typical purification procedure are shown in Figure 1. Protein concentrations were determined with a Bradford assay (Bio-Rad, Ivry, France) using bovine serum albumin as a standard and by the UV absorbance at 280 nm ($\epsilon = 18\,915\ M^{-1}\ cm^{-1}$).

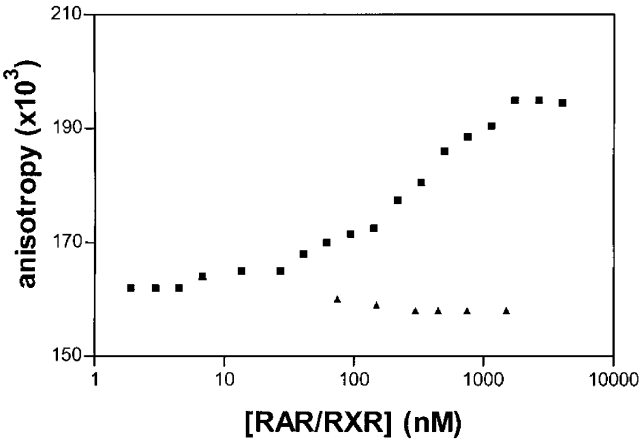


FIGURE 1: Anisotropy binding profile for binding of the RAR/RXR heterodimer to a 26 kDa fragment of the coactivator protein SRC-1 labeled on the N-terminus with the fluorescent dye Alexa 488. The SRC-1 concentration was 4 nM. The titration was performed in the presence of 5 μM 9-cisRA (■) or in the absence of ligand (▲) at 4 °C. Buffer conditions were as described in Materials and Methods.

Binding of 9-cisRA was monitored by the decrease in the intrinsic fluorescence of the heterodimer ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 340$ nm) upon titration with a ligand. The heterodimer (1×10^{-6} M) in TEGD buffer at 100 mM KCl was titrated with 9-cisRA from a concentrated solution in ethanol.

Oligonucleotides. HPLC-purified oligonucleotides were purchased from Genosys (Cambridge, U.K.). The target sequence here termed DR5 has the sequence 5′-AGCTTG-GCGCCGGGTCACCGAAAGGTCAGAATT-3′ for the sense strand. The sense strand was labeled by the supplier, and the labeling ratio was more than 80%. The fluorescein phosphoramidite was added to the 5′ end of the oligonucleotide during automated synthesis, and the fluorescein is

attached through a six-carbon linker. The sense and antisense strands were annealed by heating a 1:1 molar ratio of the unlabeled antisense strand with either a labeled or unlabeled sense strand to 85 °C for 10 min in Tris buffer in the presence of 0.1 mM EDTA and 50 mM NaCl (pH 7.5) and cooling slowly (1 °C/min) to 25 °C in a Gene Amp 2400 thermocycler (Perkin-Elmer, Norwalk, CT). Annealed oligonucleotides were stored at −80 °C.

Ligands. 9-cisRA, CD3105, and CD3106 were a kind gift from P. Ballaguer (U439, Montpellier, France). The other synthetic retinoids were a kind gift from H. Gronemeyer (IGBMC, Strasbourg, France).

Fluorescence Experiments. The buffer solution for all fluorescence measurements consisted of 20 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1 mM $ZnCl_2$, and 10% (v/v) glycerol. Binding assays were performed using a Beacon 2000 polarization instrument (Panvera, Corp., Madison, WI) regulated at 4 °C, using filters for fluorescein at an F-DR5 concentration of 2.5 nM. Anisotropy was measured successively until it stabilized, and the reported values are the average of five to eight measurements after stabilization. Binding data were analyzed using BIOEQS (*27*−*29*).

RESULTS AND DISCUSSION

Purification and Characterization of the Heterodimer. The purification of the heterodimer from *E. coli* extracts led to the rapid (within a day) isolation of a pure and homogeneous protein with only two chromatographic steps. The main purification step was a Ni^{2+} affinity chromatography for the tagged RAR in the presence of excess untagged RXR, which readily removed the majority of the contaminants; notably, the large excess of untagged RXR was eliminated during the washing steps. Gel filtration allowed us to isolate the heterodimer which was more than 90% pure as judged from the overloaded Coomassie-stained gel. The free RAR monomer and RXR tetramer were thus eliminated. The viability of the protein preparation in this study was verified by monitoring binding of 9-cisRA (*6*, *8*), and by confirming proper SRC-1 recruitment. The heterodimer was routinely found to possess 0.85−0.95 mol of binding sites for 9-cisRA per mole of protein monomer.

The interaction of SRC-$1_{570-780}$ with the RAR/RXR heterodimer was monitored by measuring changes in the steady state fluorescence anisotropy of a fluorescent probe (Alexa 488) covalently coupled to the N-terminus of the SRC-$1_{570-780}$ fragment containing the three LxxLL motifs as previously described (*30*). Indeed, interaction between ^{A488}SRC-$1_{570-780}$ and the RAR/RXR heterodimer is deduced from the increase in the anisotropy of the fluorescence emission of the fluorophore bound to the SRC-1 construct upon titration with the heterodimer in the presence of the panagonist, 9-cisRA (Figure 1). In contrast, no change in anisotropy is observed upon titration of ^{A488}SRC-$1_{570-780}$ in the absence of an agonist ligand. The binding profiles were fit using a simple model assuming a 1:1 (SRC-$1)_1$:RAR/RXR heterodimer stoichiometry and revealed a relatively high affinity of ^{A488}SRC-$1_{570-780}$ for the RAR/RXR heterodimer ($K_d \sim 300$ nM). Taken together, these findings demonstrate that we have produced a pure ΔA/B RAR/RXR heterodimer that is able to bind 9-cisRA and recruit in a ligand-dependent manner a nuclear receptor coactivator.

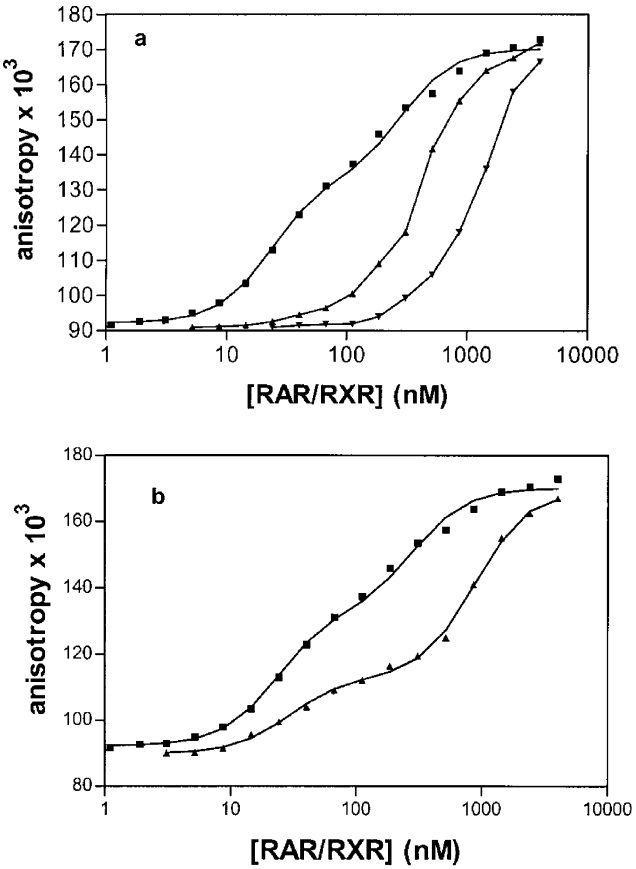
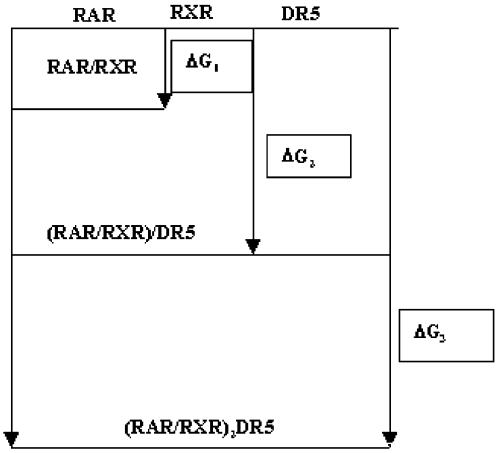


FIGURE 2: Anisotropy binding profiles for binding of the RAR/RXR heterodimer to a 5′-fluorescein-labeled DR5 target oligonucleotide. (a) Binding in the absence of ligand (■) and competition by 10-fold (▲) and 50-fold (▼) excesses of specific unlabeled DR5. (b) Binding in the absence of ligand (■) and in the presence of a 50-fold excess of nonspecific (SRBE) unlabeled DNA (▲). Lines through the points represent fits to the model described in the text. The buffer solution for all fluorescence measurements consisted of 20 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1 mM $ZnCl_2$, and 10% (v/v) glycerol. Binding assays were performed at 4 °C at an F-DR5 concentration of 2.5 nM.

Characterization of the Interaction between the RAR/RXR Heterodimer and DR5 Target DNA. The interaction of the RAR/RXR heterodimer and DNA was monitored by measuring changes in the steady state fluorescence anisotropy of a fluorescein-labeled DR5 oligonucleotide [F-5′-AGCTTGGCGCCGGGTCACCGAAAGGTCAGAATT-3′ (*31*)] upon titration with the ΔAB-RAR/RXR heterodimer. In Figure 2a is shown a representative anisotropy-based binding isotherm for binding of the purified RAR/RXR heterodimer to F-DR5 at 2.5 nM in the absence of ligand at 4 °C with 50 mM KCl in the TEDG buffer. The total fluorescence intensity did not change significantly during the titration, indicating that the anisotropy signal was not convoluted with changes in fluorescence lifetime or contaminated by scattered excitation light, and thus directly represents a molar quantity. The anisotropy profile was characterized by an initial low anisotropy plateau for the unbound DNA, followed by an anisotropy increase as the proteins form a complex with the fluorescein-labeled DNA, and concluded with a well-defined plateau.

To examine the specificity of binding, the complexes were challenged with a 10- and 50-fold excess of either unlabeled DR5 or SRBE. The SRBE is a double-stranded oligonucle-

Scheme 1: Free Energy Diagram of the Model Used To Analyze the Binding of the RAR/RXR Heterodimer to DNA



otide of 23 bp which did not contain any consensus site for the RAR/RXR heterodimer. As observed in Figure 2a, a 10- and 50-fold excess of unlabeled DR5 induced a shift in the titration curve to higher concentrations. These results clearly demonstrate the equilibrium status of the interaction between the RAR/RXR heterodimer and its target DNA under our experimental conditions. It is noteworthy that in the absence of unlabeled DNA, the binding profile presented a shoulder, instead of a simple increase in anisotropy values, thus suggesting the existence of an intermediary species. Such a bimodal aspect was clearly demonstrated by the use of an excess of unlabeled nonspecific DNA (Figure 2b). Thus, the addition of either specific or nonspecific DNA shifted the binding curve in an asymmetric fashion. The first binding event was challenged more efficiently than the second by specific DNA. This supports the conclusion that the first binding event corresponds to the specific interaction of the one RAR/RXR heterodimer with its target DNA. The situation was just the opposite when the heterodimer−F-DR5 interaction was challenged with nonspecific DNA. In this case, the effect was much more pronounced for the high-concentration region of the curve, and underscores the low specificity of this second binding event. Thus, for purposes of analysis, we will assume that this second, less specific binding event corresponds to the binding of a second heterodimer to the DNA. On the basis of the observations described above, the DNA binding anisotropy profiles of the RAR/RXR heterodimer were analyzed using the numerically based program BIOEQS (*27*−*29*) according to a model of two heterodimers binding to the DNA. In addition to the free monomers and free DNA species, the model included the heterodimer bound to the DNA and a final species corresponding to two heterodimers bound to the DNA. The free energies, which were the parameters in the fit and which are given in Scheme 1, correspond to free energies of formation of the complexes from the fundamental elements. Thus, ΔG_1 corresponds to the free energy of heterodimerization.

$$\Delta G_1 = -RT \ln[([\text{RAR}][\text{RXR}])/[\text{RAR/RXR}]] \quad (1)$$

The value of ΔG_2 is the free energy of formation of the RAR/RXR−DR5 complex from the free monomers and the DNA.

$$\Delta G_2 = -RT \ln[([\text{RAR}][\text{RXR}][\text{DR5}])/[\text{RAR/RXR}-\text{DR5}]] \quad (2)$$

The free energy for heterodimer binding to DR5 is therefore equal to the difference between ΔG_2 and ΔG_1, according to Scheme 1. ΔG_3 corresponds to the free energy of formation of the complex of DR5 with two heterodimers from the free monomers and the DNA.

$$\Delta G_3 = -RT \ln[([\text{RAR}]^2[\text{RXR}]^2[\text{DR5}])/[(\text{RAR/RXR})_2-\text{DR5}]] \quad (3)$$

The free energy for the second heterodimer binding to the once-bound complex is $\Delta G_3 - \Delta G_2$.

Given values for the total concentrations of all of the elements (RAR, RXR, and DR5) at each concentration point for each titration and values for the free energies of formation of the complex species from those elements (ΔG_1−ΔG_3), the solver in the program BIOEQS solves this set of nonlinear equations for the species concentration vector using a constrained optimization algorithm and incorporating the total elemental concentration constraints as Langrange multipliers (*27*−*29*). Using the values for the anisotropy of each of the proposed species, BIOEQS then calculates the anisotropy curve for each titration and then uses a Marquardt−Levenberg algorithm to adjust all of the parameter values (anisotropies and free energies) to best fit the data.

Because the titrations were carried out over a concentration range extending down to 1 nM, it was necessary to consider that at these low concentrations, the heterodimer itself could dissociate into monomers, and that heterodimerization could be coupled to DNA binding (i.e., that the first DNA binding event could be cooperative). To take into account this possibility, the heterodimer could not be regarded as a single molecular element. Thus, the system was considered to consist of three distinct, fundamental elements, RAR monomers, RXR monomers, and the target oligonucleotide. Since both the concentration of RAR and that of RXR change simultaneously over the course of the titration, each curve was analyzed globally with each data point (each concentration of RAR) corresponding to one data set, for which the corresponding concentration of RXR was specified. This is equivalent to a titration along the diagonal in an $x-y$ plane, in which the x-axis corresponds to the RAR concentration and the y-axis to the RXR concentration.

We note that the uncertainties in the values of the recovered parameters were estimated at the 67% confidence limit (one standard deviation) using rigorous confidence limit testing in which, for each value for each parameter tested, the data were refit, allowing all of the other parameters to vary. This method of rigorous confidence limit testing thus allows for the evaluation of the correlation between individual parameters in the fit. This provides a much more thorough evaluation of the confidence one can have in any parameter value than is typically obtained from the diagonal elements of the error matrix (*32*, *33*).

Analysis of the curves in Figure 2 for the titration of DR5 with the heterodimer in the absence and presence of a nonspecific competitor at a 50-fold excess according to the above-described model revealed that the first binding event is highly cooperative because the recovered free energy of dimer formation (ΔG_1) was found to be near zero, i.e.,

Table 1: Energetic Parameters Recovered from the RAR/RXR−DR5 Anisotropy Binding Profiles

	ΔG_2[a] (kcal/mol)	ΔG_3 (kcal/mol)
no ligand	20.6 ± 0.2	38.2
no ligand with a 50-fold excess of nonspecific DNA	20.3 ± 0.3	36.6
9-cisRA	20.7 ± 0.2	38.4
CD3152	20.3 ± 0.3	37.9
CD3153	18.7 ± 0.1	36.5
CD3105	17.6 (−2.2, +0.3)	35.1
CD3106	17.8 (−0.3, +0.1)	35.1
CD3105dn	−	34.2

[a] The 67% confidence limits on the values of ΔG_2 (the free energy of formation of the specific RAR/RXR−DR5 complex) were determined by rigorous confidence limit testing.

essentially undetermined by the data. This cooperativity is actually directly apparent in the raw data; the first binding event occurs over approximately one log unit [10−100 nM heterodimer, Figure 2b (▲)], whereas a normal two-body binding event occurs over 1.908 log units of concentration. From this cooperativity, we can conclude that the interaction involves at least a three-body interaction, the simplest of which is RAR/RXR heterodimerization coupled to binding to the target DNA. Since this interaction is specific (challenged by specific, but not by nonspecific, DNA; Table 1) and only the RAR/RXR heterodimer binds specifically to the DR5 target, not RXR or RAR alone (*18*, *19*), the assumption that the first binding event corresponds to formation of the DR5−heterodimer complex appears to be highly plausible. Additional support for this assumption comes from the observation that RAR specific ligands have a large effect on the formation of this complex (see below). Since RAR is known to exist only as monomers, even at concentrations much higher that those used in the titrations presented here (indeed we separate RAR monomers from the heterodimer in the gel filtration step of the purification), and since RXR alone is known to exhibit no interaction with DR5 under these conditions (*22*), we conclude that the first complex formed includes both RAR and RXR. While it is conceivable that the first binding event could correspond to the binding of two heterodimers to the DNA rather than one, we feel that this is unlikely. First, the change in anisotropy is quite small for this event, and second, one would not expect that RAR/RXR heterodimers would associate at these low concentrations. Thus, we feel it is justified to consider that the first binding event corresponds to the formation of the DR5−heterodimer complex.

The high degree of cooperativity means that the free energy of dimer formation is significantly lower in absolute value than the free energy of dimer binding to DNA, and thus that little free heterodimer is populated at equilibrium in the concentration range (~20 nM) where DNA binding occurs. Thus, any heterodimer that is formed is bound to DNA, and dimerization and DNA binding are highly coupled thermodynamically. We note that we could have also modeled a monomer-bound DNA intermediate, rather than the free heterodimeric intermediate. In either case, the result would have been the same, in the sense that the cooperativity of this first binding event would have been very high. An equilibrium measurement cannot distinguish between a mechanism involving an intermediate free dimer and one involving a monomer−DNA complex.

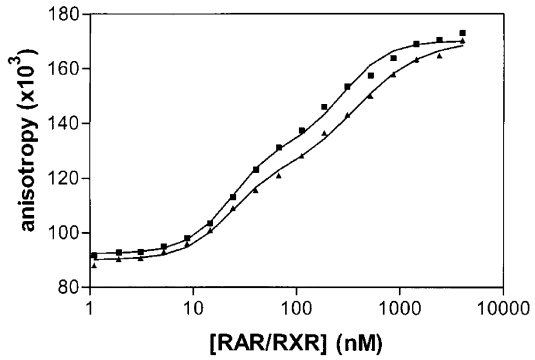


FIGURE 3: Effect of the panagonist ligand, 9-cisRA, on the RAR/RXR−DR5 interaction. Anisotropy profile in the absence of ligand (■) and in the presence of 5 μM 9-cisRA (▲). Lines through the points represent fits to the model described in the text. Titration conditions were as described in the legend of Figure 2.

The total free energy of formation of the heterodimer−DR5 complex from the free monomers and free DNA (ΔG_2) was found to be 20.6 kcal/mol, while the energy for the second binding event, binding ($\Delta G_3 - \Delta G_2$) to this complex, is significantly lower, 17.6 kcal/mol (Table 1). This difference of 3 kcal/mol corresponds to a difference in concentration range for the midpoints of the two binding events of >2 log units, a very large difference in terms of typical biological control. In the presence of nonspecific DNA, the apparent free energy of formation of the heterodimer−DR5 complex remained essentially unchanged (20.3 kcal/mol), whereas that of formation of the second complex was destabilized by 1.6 kcal/mol, consistent with the nonspecific nature of this interaction.

Effects of Ligands on the Interaction between the RAR/RXR Heterodimer and DNA. As shown in Figure 3, the DNA binding profile of the RAR/RXR heterodimer in the presence of the panagonist ligand, 9-cisRA, showed only very small differences compared to that of the unliganded protein. Analysis of this binding profile yielded the same free energy of formation of the heterodimer/DNA complex as in the absence of ligand (Table 1). Binding remained highly cooperative in this case as well, and competition by specific and nonspecific DNA was identical to that found in the absence of ligand (data not shown).

In contrast, RAR specific antagonist ligands had a very marked effect on the heterodimer DNA binding profiles (Figure 4). We have chosen to investigate two C-1-substituted acetylenic retinoids that exhibit potent antagonism of retinoic acid receptor (RAR)-mediated transactivation (*34*), one of which, CD3105, exhibits neutral antagonism and the other of which, CD3106, acts as a reverse agonist. We have also investigated the effects of two other RAR specific antagonists, CD3152 and CD3153. Two distinct effects of these different ligands on the profiles of heterodimer−DNA binding are observed. First, all of the antagonist ligands, with the exception of CD3152, shifted the binding profiles to significantly higher heterodimer concentrations. The antagonists CD3153 and CD3106 led to similar shifts in the binding curves, although the apparent cooperativity (steepness of the curve) was greater in the presence of CD3106. The second observed effect is a small yet significant increase in the absolute value of the anisotropy at saturation observed in the presence of all of the antagonists with the exception of

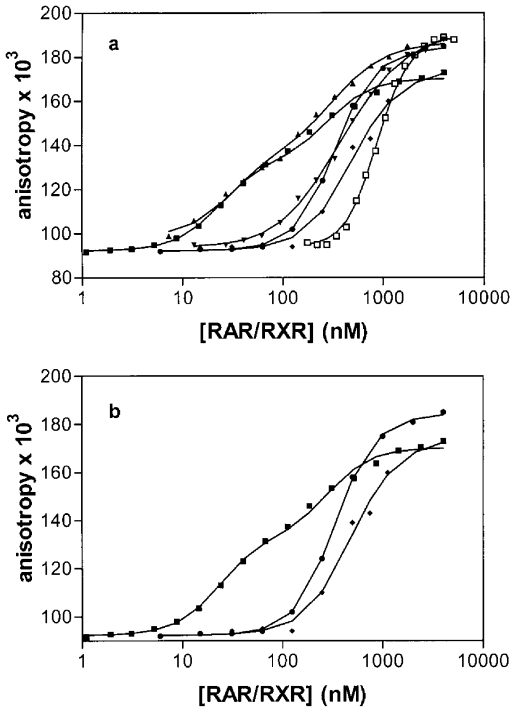


FIGURE 4: Effect of antagonist ligands on the RAR/RXR−DR5 interaction. (a) Profiles in the presence of all of the antagonist ligands are presented. The symbols correspond to titrations in the absence of ligand (■) and in the presence of saturating CD3152 (▲), CD3153 (▼), CD3105 (◆), CD3106 (●), and CD3105 (□) using the dominant negative (DN) heterodimer construct. (b) Comparison of the profiles obtained in the absence of ligand (■) and in the presence of saturating CD3105 (◆) and CD3106 (●). In particular, the differences in the final plateau anisotropy values for CD3105 and CD3106 should be noted. All ligands were used at 10 μM to ensure saturation of the RAR even at the highest protein concentrations. Lines through the points represent fits to the model described in the text. Titration conditions were as described in the legend of Figure 2.

CD3105. In the presence of the latter, the high-concentration plateau anisotropy was identical to that measured in the absence of ligand. Interestingly, titration in the presence of the CD3105 antagonist of the heterodimer containing a dominant negative mutant of RAR in which the activation helix 12 was deleted resulted in a high-concentration plateau anisotropy value for the titrations in the presence of CD3105 that was identical to that observed for the wild-type protein in the presence of the other tested antagonists. The fact that the differences observed in plateau values are so small (20 $\times$ 10^{-3} anisotropy unit) renders the attribution of its origin by time-resolved anisotropy impractical, and suggests strongly that these differences arise from differences in the local mobility of the probe, rather than in the size of the protein−DNA complex at saturation. Despite the fact that these differences are small, they are well outside the uncertainty of the measurements, and reveal that the protein−DNA complexes in the presence of the antagonists present structural and dynamic differences that are sensed by the fluorophore. It is interesting to note that while the antagonist CD3105 acts apparently as a neutral antagonist, CD3106 exhibits reverse agonist effects (*34*). Perhaps the fact that the anisotropy plateau value was different for the complexes in the presence of the two different antagonists reflects differences in their structural and dynamic properties that can be linked to differences in function.

Quantitative analysis of the binding profiles according to the model used above for the titrations in the absence of ligand and in the presence of 9-cisRA revealed a slight destabilization by the antagonist CD3152 of the heterodimer−DR5 complex (−0.4 kcal/mol) compared to the case of the agonist, 9-cisRA. As is apparent from examination of the profiles, much stronger effects were observed for the other antagonists, CD3153, CD3106, and CD3105, which destabilized the specific heterodimer−DR5 complex by 2−3 kcal/mol (see Table 1). The cooperativity observed for the titration with the dominant negative mutant of the RAR in the heterodimer in the presence of CD3105 was so high that the free energy of formation of the specific heterodimer−DR5 complex could not be resolved. However, the total free energy of formation of the heterotetramer−DR5 complex was diminished by 4 kcal/mol, most of which is likely, as for the other antagonists, to be imputable to formation of the specific heterodimer−DR5 complex.

CONCLUSIONS

We have investigated here the effect of agonist and antagonist ligands on the apparent affinity of the RAR/RXR heterodimer for a DR5 target oligonucleotide. We have shown first that the heterodimer binds in two steps to the target DNA. Competition by specific and nonspecific unlabeled DNA allows us to conclude that the first binding event (high affinity, high specificity, and high cooperativity) is likely to correspond to the specific binding of one heterodimer to DNA. The presence of RAR in the complexes is confirmed by the crucial effect of RAR specific ligands on the protein−DNA interaction. Moreover, specific binding to DR5 is only observed for the RAR/RXR heterodimer, not the RAR monomer or the RXR homodimer. The cooperativity of the binding of the first heterodimer implies that heterodimerization is coupled to DNA binding, through either prior dimerization, an intermediate monomer−DNA complex, or both. The second binding event, exhibiting low affinity and specificity, likely corresponds to the binding of a second heterodimer. This assumption concerning the binding of a second heterodimer to the preformed protein−DNA complex is supported by the fact that RXR exhibits a tendency to form tetramers in solution (*6*), and it is conceivable that dimers of RAR/RXR heterodimers could form in a similar fashion, albeit with lower affinity. Since at these high concentrations the heterodimer is the most stable species (indeed, we purify it under these conditions), it is highly unlikely that either the RAR or RXR would dissociate from the heterodimer to bind alone to the DNA nonspecifically. Thus, although this binding event is not of primary interest, we feel it is justified to assume that it corresponds to the formation of a complex involving a second heterodimer. Formally, even more heterodimers could be involved, but the data are adequately described by only one additional heterodimer.

The most interesting observations of this study involve the effect of ligands on the specific interaction of the heterodimer with DR5. While it is quite clear that the panagonist 9-cisRA has little effect on the apparent affinity of the heterodimer for DNA, the RAR antagonists lead to very striking decreases in the level of overall DNA binding. Does this strong negative effect of antagonists arise from a direct transfer of ligand binding information from the LBD

to the DBD via the linker region, or is it coupled to DNA binding through a destabilization of the heterodimer? Given the well-known allosteric effects of RAR ligands on RXR function (*19*, *20*, *35*), we can conclude that at least in terms of the LBD, ligand binding can have an effect on protein−protein interactions. Depoix and co-workers (*36*) have reported GST pulldown experiments that suggest that RAR antagonist may destabilize the heterodimer, although the ligand rather than the protein concentration was varied in these experiments. Studies of the TR/RXR heterodimer revealed a key role of the linker domain in ligand binding, DNA recognition, and heterodimerization (*37*). Without structural data for liganded and apo full-length heterodimers, it will be difficult to ascertain which mechanism is vital for this strong effect of antagonist on DNA binding, particularly since kinetic studies on these fragile proteins are not practical. Nonetheless, the studies presented here have clearly demonstrated that the heterodimer liganded by a number of RAR specific antagonists binds to the DR5 target with considerably lower affinity. Since the direct action of nuclear receptors requires that they be bound to their response elements, these results suggest that in addition to coactivator dissociation and/or corepressor recruitment, RAR antagonist ligands act to impede receptor interaction with DNA. Thus, antagonist efficiency may depend in part upon the degree to which certain compounds disfavor heterodimer−DNA interactions.

ACKNOWLEDGMENT

We acknowledge Patrick Ballaguer and Jean-Claude Nicolas of INSERM 439 for the gift of the RAR specific antagonist ligands and for many helpful discussions.

REFERENCES

1. Chambon, P. (1996) A decade of molecular biology of retinoid receptors, *FASEB J. 10*, 940−954.
2. Kliewer, S. A., Emsono, K., Mangelsdorf, D. J., and Evans, R. M. (1992) Retinoid X receptor interacts with the nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling, *Nature 355*, 446−449.
3. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J. Y., Staub, A., Gernier, J. M., and Mader, S. (1992) Purification, cloning and RXR identity in the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently, *Cell 68*, 377−395.
4. Zhang, X. G., Hoffman, B., Tran, P. B., Graupner, G., and Pfahl, M. (1992) Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors, *Nature 355*, 441−446.
5. Durand, B., Saunders, M., Leroy, P., Leid, M., and Chambon, P. (1992) All-trans and 9-cis retinoic acid induction of CRABPII transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs, *Cell 71*, 73−85.
6. Kersten, S., Kelleher, D., Chambin, P., Gronemeyer, H., and Noy, N. (1995) Retinoid X receptor a forms tetramers in solution, *Proc. Natl. Acad. Sci. U.S.A. 92*, 8645−8649.
7. Minucci, S., Leid, M., Toyama, R., Saint-Jeannet, J.-P., Peterson, V. J., Horn, V., Ishmael, J. E., Chattacharyya, N., Dey, A., Dawid, I. B., and Ozato, K. (1997) Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid dependent gene expression, *Mol. Cell. Biol. 17*, 644−655.
8. Chen, J.-Y., Clifford, J., Zusi, C., Starrett, J., Tortolani, D., Ostrowski, J., Reczek, P. R., Chambon, P., and Gronemeyer, H. (1996) Two distinct actions of retinoid receptor ligands, *Nature 382*, 819−822.
9. Freedman, L. P. (1999) Increasing complexity of coactivation in nuclear receptor signaling, *Cell 97*, 5−8.
10. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) Nuclear receptor co-regulators: Cellular and molecular biology, *Endocr. Rev. 20*, 321−344.
11. Perlmann, T., Umesono, K., Rangarajan, P. N., Forman, B. M., and Evans, R. M. (1996) Two distinct dimerization interfaces differentially modulate target gene specificity of nuclear hormone receptors, *Mol. Endocrinol. 10*, 958−966.
12. Katahira, M., Knegtel, R. M., Boelens, R., Eib, D., Schilthuis, J. G., van der Saag, P. T., and Kaptein, R. (1992) Homo- and heteronuclear NMR studies of the human retinoic acid receptor beta DNA-binding domain: sequential assignments and identification of secondary structure elements, *Biochemistry 31*, 6474−6480.
13. Rastinejad, F., Perlmann, T., Evans, R. M., and Sigler, P. B. (1995) Structural determinants of nuclear receptor assembly on DNA direct repeats, *Nature 375*, 203−211.
14. Zhao, Q., Chasse, S. A., Devarakonda, S., Sierk, M. L., Ahvazi, B., and Rastinejad, F. (2000) Structural basis of RXR-DNA interactions, *J. Mol. Biol. 296*, 509−520.
15. Rastinejad, F., Wagner, T., Zhao, Q., and Khorasanizadeh, S. (2000) Structure of the RXR-RAR DNA-binding complex on the retinoic acid response element DR1, *EMBO J. 19*, 1045−1054.
16. Shaffer, P. L., and Gewirth, D. T. (2002) Structural basis of VDR-DNA interactions on direct repeat response elements, *EMBO J. 21*, 2242−2252.
17. Bourguet, W., Vivat, V., Wurtz, J. M., Chambon, P., Gronemeyer, H., and Moras, D. (2000) Crystal structure of a heterodimeric complex of RAR and RXR ligand-binding domains, *Mol. Cell 5*, 289−298.
18. Rastinejad, F. (2001) Retinoid X receptor and its partners in the nuclear receptor family, *Curr. Opin. Struct. Biol. 11*, 33−38.
19. Khorasanizadeh, S., and Rastinejad, F. (2001) Nuclear-receptor interactions on DNA-response elements, *Trends Biochem. Sci. 26*, 384−390.
20. Westin, S., Kurokawa, R., Nolte, R. T., Wisely, G. B., McInerney, E. M., Rose, D. W., Milburn, M. V., Rosenfeld, M. G., and Glass, C. K. (1998) Interactions controlling the assembly of nuclear receptor heterodimers and co-activators, *Nature 395*, 199−202.
21. Germain, P., Iyer, J., Zechel, C., and Gronemeyer, H. (2002) Co-regulator recruitment and the mechanism of retinoic acid receptor synergy, *Nature 415*, 187−192.
22. Egea, P. F., Rocherl, N., Birck, C., Vacehtte, P., Timmins, P. A., and Moras, D. (2001) Effects of ligand binding on the association properties and conformation in solution of retinoic acid receptors RXR and RAR, *J. Mol. Biol. 307*, 557−576.
23. Grillo, A. O., Brown, M. P., and Royer, C. A. (1999) Probing the physical basis for *trp* repressor-operator recognition, *J. Mol. Biol. 287*, 539−554.
24. Mader, S., Chen, J. Y., Chen, Z., White, J., Chambon, P., and Gronemeyer, H. (1993) The patterns of binding of RAR, RXR and TR homo- and heterodimers to direct repeats are dictated by the binding specificities of the DNA binding domains, *EMBO J. 12*, 5029−5041.
25. Iyer, J., Bonnier, D., Granger, F., Iltis, C., Andry, V., Schultz, P., and Gronemeyer, H. (1999) Versatile co-purification procedure for rapid isolation of homogeneous RAR-RXR heterodimers, *Protein Expression Purif. 16*, 308−314.
26. Chen, Z. P., Iyer, J., Bourguet, W., Held, P., Mioskowski, C., Lebeau, L., Noy, N., Chambon, P., and Gronemeyer, H. (1998) Ligand- and DNA-induced dissociation of RXR tetramers, *J. Mol. Biol. 275*, 55−65.
27. Royer, C. A., Smith, W. R., and Beechem, J. M. (1990) Analysis of Binding in Macromolecular Complexes: A Generalized Numerical Approach, *Anal. Biochem. 192*, 287−294.
28. Royer, C. A., and Beechem, J. M. (1992) Numerical Analysis of Binding Data: Advantages, Practical Aspects and Implications, *Methods Enzymol. 210*, 481−505.
29. Royer, C. A. (1993) Improvements in the Numerical Analysis of Thermodynamic Data from Biomolecular Complexes, *Anal. Biochem. 210*, 91−97.
30. Margeat, E., Poujol, N., Boulahtouf, A., Chen, Y., Muller, J., Gratton, E., Cavaillès, V., and Royer, C. A. (2001) The estrogen receptor α dimer binds a single SRC-1 coactivator molecule with an affinity dictated by agonist structure, *J. Mol. Biol. 306*, 433−454.
31. Kersten, S., Dawson, I., Lewis, B. A., and Noy, N. (1996) Individual subunits of heterodimers comprised of retinoic acid and retinoid X receptors interact with their ligands independently, *Biochemistry 35*, 3816−3824.

32. Johnson, J. L., and Fount, L. M. (1992) Parameter estimation by least squares analysis, *Methods Enzymol. 210*, 1−36.
33. Beechem, J. M. (1992) Global analysis of biochemical and biophysical data, *Methods Enzymol. 210*, 37−53.
34. Klein, E. S., Pino, M. E., Johnson, A. T., Davies, P. J., Nagpal, S., Thacher, S. M., Krasinski, G., and Chandraratna, R. A. (1996) Identification and functional separation of retinoic acid receptor neutral antagonists and inverse agonists, *J. Biol. Chem. 271*, 22692−22696.
35. Vivat, V., Zechel, C., Wurtz, J.-M., Bourguet, W., Kagechika, H., Umemiya, H., Shudo, K., Moras, D., Gronemeyer, H., and Chambon, P. (1997) A mutation mimicking ligand-induced conformational change yields a constitutive RXR that senses allosteric effects in heterodimers, *EMBO J. 16*, 5697−5709.
36. Depoix, C., Delmotte, M.-H., Formstecher, P., and Lefebvre, P. (2001) Control of retinoic acid receptor heterodimerization by ligand-induced structural transitions, *J. Biol. Chem. 276*, 9452−9459.
37. Miyamoto, T., Kakizwa, T., Ichikawa, K., Nishio, S., Takeda, T., Suzuki, S., Kaneko, A., Kmagai, M., Mori, J., Yamashita, K., Sakuma, T., and Hashizumi, K. (2001) The role of the hinge domain in heterodimerization and specific DNA recognition by nuclear receptors, *Mol. Cell. Endocrinol. 181*, 229−238.

BI027056L