On the Role of Ligand in Retinoid Signaling: Positive Cooperativity in the Interactions of 9-*cis* Retinoic Acid with Tetramers of the Retinoid X Receptor†

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ABSTRACT: Previously, we have shown that the retinoid X receptor (RXR) forms tetramers with a high affinity and that interactions of the receptor with its ligand, 9-*cis* retinoic acid (9cRA), result in dissociation of protein tetramers. Here it is shown by fluorescence anisotropy studies that ligand-induced tetramer dissociation displays a pronounced positive cooperativity. The binding affinity of RXR for 9cRA at low saturation levels of the receptor with ligand was found to be significantly weaker than the affinity observed at higher levels of saturation. In addition, the rate of dissociation of 9cRA from RXR was found to be faster at low *vs.* high saturation levels of the receptor. These data suggest that the observed positive cooperativity of the ligand-induced dissociation of RXR tetramers stems from positive cooperativity in binding of 9cRA by the receptor. Kinetic studies showed that dissociation of RXR tetramers upon ligand binding is a rapid reaction characterized by a $t_{1/2}$ of 80 ms, which is about 5 orders of magnitude faster than the rate of dissociation in the absence of ligand. The data indicate that the oligomeric state of RXR is tightly regulated by the precise concentrations of 9cRA and that it rapidly responds to changes in the ligand's concentrations. These findings further substantiate the hypothesis that modulation of the oligomeric state of RXR by 9cRA is an important regulatory step in the pathway by which retinoids affect gene transcription.

Retinoic acids are lipophilic hormones that can affect gene transcription by direct interactions with two classes of transcription factors: the retinoic acid receptors (RARs) which bind all-*trans* and 9-*cis* retinoic acids (tRA and 9cRA), and the retinoid X receptors (RXRs) which bind 9cRA exclusively (for reviews see Glass, 1994; Giguère, 1994; Mangelsdorf *et al.*, 1994). These proteins belong to a super-family of nuclear hormone receptors that also includes the vitamin D receptors, the thyroid hormone receptors, and the peroxisome proliferators activated receptors. Transcriptional activation occurs by interaction of these receptors with specific response elements in the promoter region of target genes. Both RARs and RXRs can bind to cognate DNA as homodimers, and RXRs can also form heterodimers with other members of the receptor super-family, usually resulting in tighter interactions with DNA (Yu *et al.*, 1991; Durand *et al.*, 1992; Leid *et al.*, 1992; Zhang *et al.*, 1992). RXR thus functions as a general binding partner that communicates between converging signaling pathways (Glass *et al.*, 1994).

It is well documented that ligands are important for activation of gene transcription by retinoid receptors. However, little information is currently available regarding the exact role of ligand binding for receptor function. It was proposed that 9cRA functions by strengthening the formation of RXR homodimer both in solution and when bound to DNA, thereby diverting RXR away from the heterodimeric interactions with other nuclear receptors (Leid 1994; Zhang *et al.*, 1994). However, recent evidence suggests that this model may not be correct (see discussion in Chen *et al.*, 1994), and hence, the role of the ligand in retinoid signaling remains elusive.

We have recently demonstrated that in solution, RXR forms tetramers with a high affinity (Kersten *et al.*, 1995a), and that binding of 9cRA to the receptor results in dissociation of the tetramers to dimers and monomers (Kersten *et al.*, 1995b). These findings led to the suggestion that signaling by 9cRA may occur *via* modulation of the oligomeric state of RXR (Kersten *et al.*, 1995b).

The data in the present manuscript demonstrate that dissociation of RXR tetramers induced by binding of 9cRA is a rapid process that is characterized by pronounced positive cooperativity. It is shown further that cooperativity in ligand-induced tetramer dissociation is likely to originate from positive cooperativity in ligand binding by the receptor. These findings suggest that dissociation of RXR tetramers is tightly regulated by the precise concentrations of the ligand and further substantiate the hypothesis that modulation of the oligomeric state of RXR by 9cRA is an important regulatory step in the pathway by which retinoids affect gene transcription.

EXPERIMENTAL PROCEDURES

Ligands. 9cRA was a gift from Hoffman La-Roche (Nutley, NJ). All-*trans* retinoic acid was purchased from Kodak.

Protein. In this study, RXRα lacking the N-terminal A/B domain (RXRαΔAB) was used. The text of this paper refers to this truncated protein as RXR. Protein was synthesized by overexpression in *E. coli* using the pET16b expression system as previously described (Chen *et al.*, 1994). This receptor preparation was previously shown to bind its ligand, 9cRA, with high affinity, to form homo- and heterodimers, and to associate properly with cognate DNA

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(Chen *et al.*, 1994). Viability of protein preparations in this study was verified by monitoring binding of 9cRA (Chen *et al.*, 1994; Kersten *et al.*, 1995a), and by confirming proper DNA recognition. RXR was routinely found to possess 0.7−0.85 mol binding sites for 9cRA per mole of protein. Protein concentrations were determined by the Bradford assay (BioRad) using bovine serum albumin as a standard.

Unilamellar Vesicles. Small unilamellar vesicles of dioleoylphosphatidylcholine (DOPC) or of DOPC containing 2 mol% of the fluorescent probe 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine (PY-PC) were prepared by sonication. DOPC (or DOPC and PY-PC) was (were) dissolved in chloroform, the solvent was evaporated under argon, and the mixture dried for additional 2 hr under vacuum. Lipids were suspended in buffer A (10 mM Hepes at pH 8.0, 0.1 mM EDTA, 0.5 mM DTT, 400 mM KCl, 5% glycerol) containing 100 mM KCl. The suspension was sonicated to clarity using a Heat-System sonicator, and centrifuged at 100 000*g* for 15 min to pellet multilamellar vesicles. Lipid concentrations were determined by the phosphorus content (Dittmer & Wells, 1969).

Fluorescence Studies. These were carried out using a SPEX (Metachen, NJ) fluorolog-2 spectrofluorometer equipped with Glan-Thompson polarizers. Rapid mixing was achieved with a Hi-Tech (England) stopped flow accessory in conjunction with the fluorometer.

Fluorescence Anisotropy Titrations of RXR with 9cRA. RXR in buffer A was covalently labeled with the fluorescent probe fluorescein as previously described (Kersten *et al.*, 1995a). Labeled RXR (1 μM) in buffer A containing 100 mM KCl was titrated with 9cRA from a concentrated solution in ethanol. Final ethanol concentration did not exceed 2%. At each point, the fluorescence anisotropy (λ_{ex} = 491 nm, λ_{em} = 516 nm) was measured four times to obtain a mean.

Partitioning of 9cRA between RXR and Unilamellar Lipid Vesicles. The relative binding affinities of RXR for 9cRA at different saturation levels of the receptor were measured by monitoring the partitioning of 9cRA between RXR and unilamellar vesicles containing the fluorescent probe PY-PC. The fluorescence emission spectrum of PY-PC overlaps with the absorption spectrum of 9cRA, and the presence of this ligand in close proximity to the probe results in quenching of probe fluorescence. A standard curve describing the relation between the amount of 9cRA in the lipid bilayers and the fluorescence of the probe was constructed by titrating the vesicles with 9cRA and monitoring PY-PC fluorescence (λ_{ex} = 330 nm, λ_{em} = 380 nm). To measure partitioning of 9cRA between vesicles and RXR, vesicles (20 μM lipids) and RXR (1 μM) were mixed, the mixture was titrated with 9cRA, and the fluorescence of the probe was measured. The amount of 9cRA in the vesicles was obtained from the change in the fluorescence of the probe following each titration step by using the standard curve. Given the low solubility of 9cRA in water, it could be assumed that the remainder of the ligand was bound to RXR.

Kinetics of Transfer of 9cRA from RXR to Unilamellar Vesicles of DOPC. RXRαΔAB (2 μM) was complexed with 9cRA at a ligand/protein molar ratio of 0.1 or 0.7. The protein was mixed with vesicles of DOPC (100−5000 μM lipids) using the stopped-flow accessory. Transfer of 9cRA from RXR to vesicle was monitored by the time-dependent increase in the intrinsic fluorescence of the protein (λ_{ex} = 280 nm; λ_{em} = 340 nm).

Kinetics of Dissociation of RXR Tetramers Following Dilution of the Protein. Fluorescein-labeled RXR (13 μM) was diluted into a cuvette to a final concentration of 50 nM. The fluorescence anisotropy of the labeled protein (λ_{ex} = 491 nm; λ_{em} = 516 nm) was measured immediately after dilution and subsequently at regular intervals. For each time point, the fluorescence anisotropy was measured four times to obtain a mean.

Kinetics of Ligand-Induced Dissociation of RXR Tetramers. Fluorescein-labeled RXR (2 μM) was mixed with either 9cRA or tRA (2 μM in buffer A containing 100 mM KCl) using the stopped-flow accessory. The volume ratio of the two solutions was 1/1. The fluorescence of the labeled protein (λ_{ex} = 491 nm, λ_{em} = 516 nm) was measured until equilibrium was approached.

RESULTS

Ligand-Induced Dissociation of RXR Tetramers. We have recently demonstrated that RXR exists in solution as a tetramer (Kersten *et al.*, 1995a) and that binding of 9cRA to the receptor results in dissociation of RXR tetramers to dimers (Kersten *et al.*, 1995b). Oligomerization of RXR could be followed by monitoring the fluorescence anisotropy of the receptor covalently labeled with a fluorescent probe. Fluorescence anisotropy is a sensitive measure of the rotational volume of a fluorescent molecule and can be used to follow variations in the size of the fluorophore such as those that accompany association−dissociation reactions (e.g., Fernando & Royer, 1992; Noy *et al.*, 1992; Kwon & Churchich 1994). Thus, it was previously demonstrated that association of RXR into dimers and tetramers can be quantitatively studied by monitoring the fluorescence anisotropy of the receptor covalently labeled with the fluorescent probe fluorescein, and that ligand-induced dissociation of RXR tetramers occurred concomitantly with a significant decrease in this parameter (Kersten *et al.*, 1995a,b).

In order to examine further the ligand-induced dissociation of RXR tetramers, the fluorescence anisotropy of fluorescein-labeled RXR was followed upon titration of the receptor with 9cRA. The titration was carried out at a receptor concentration of 1 μM at which the predominant protein species are RXR tetramers (Kersten *et al.*, 1995). Upon addition of ligand, the fluorescence anisotropy decreased, reflecting tetramer dissociation (Figure 1, closed circles). Dissociation was complete at a ligand/protein ratio of about 1, demonstrating that the observed process was related to high-affinity ligand binding by RXR. In addition to the decrease in anisotropy, dissociation of RXR tetramers was also accompanied by a decrease in the total fluorescence of the labeled protein (Figure 1, open circles). This did not stem from variations in the fluorescence lifetime of the probe as was ascertained by the constant value of the fluorescence lifetime of labeled RXR tetramers and dimers in the absence and in the presence of 9cRA (Kersten *et al.*, 1995a,b). The observed changes in the fluorescence of the labeled protein that accompanied ligand-induced dissociation are likely to reflect changes in the local environment of the probe probably due to the significant conformational differences between RXR tetramers and dimers (Kersten *et al.*, 1995b).

The decrease in the total fluorescence and in the fluorescence anisotropy of labeled RXR followed a sigmoidal curve, a behavior that is indicative of pronounced positive coop-

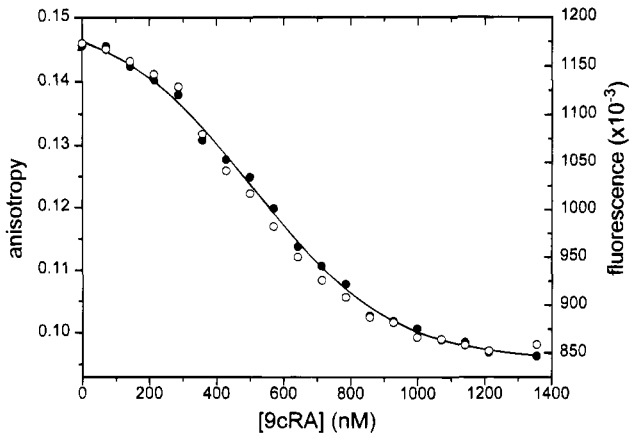

FIGURE 1: Effect of 9cRA on total fluorescence and on fluorescence anisotropy of fluorescein-labeled RXR. Fluorescein-labeled RXR (1 μM) was titrated with 9cRA from a concentrated solution in ethanol. Fluorescence anisotropy (closed circles) and total fluorescence (open circles) were measured until a constant value was reached. Line represents the fit of fluorescence anisotropy values according to the sigmoid fitting function of the MicroCal Origin software program.

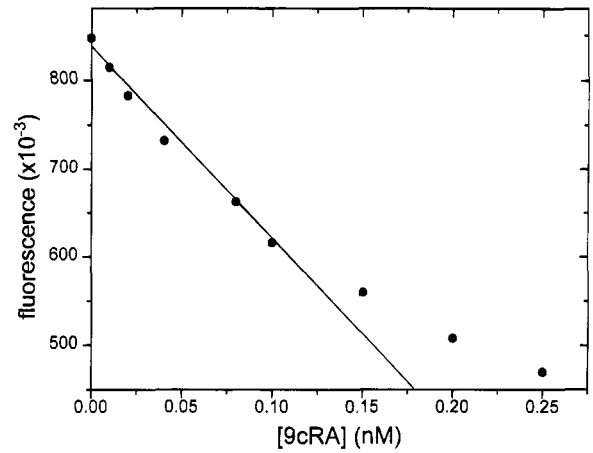

FIGURE 2: Fluorescence of PY-PC incorporated in DOPC vesicles as a function of the concentration of 9cRA in the vesicles. Small unilamellar vesicles of DOPC (20 μM) containing the fluorescent lipid probe PY-PC were titrated with 9cRA from a concentrated solution in ethanol. The fluorescence of the probe (λ_{ex} = 330 nm; λ_{em} = 380 nm) was measured. The range in which the fluorescence linearly related to the concentration of 9cRA was used as a calibration curve.

erativity in tetramer dissociation. This cooperativity in the ligand-induced tetramer dissociation could arise from cooperativity in binding of 9cRA to the different receptor subunits. Alternatively, binding of 9cRA to the individual subunits could proceed with similar affinities and the observed cooperativity could reflect cooperativity in the actual process of dissociation.

Relative Affinity of RXR for 9cRA at Different Saturation Levels of the Receptor. To differentiate between the alternative origins for the cooperativity in dissociation of RXR tetramers, the pattern of binding of 9cRA to RXR should be examined. Assays for binding of hydrophobic ligands to soluble proteins are, however, complicated by the tendency of such ligands to adhere to matrixes that are used to separate free from bound species. Fluorescence-based methodologies that do not require physical separation of free from bound ligand have been developed and widely used to monitor binding of retinoids to a variety of proteins including RXR (e.g., Cogan *et al.*, 1976; Ong & Chytil, 1980; Chen & Noy, 1994; Chen *et al.*, 1994). However, because of the limits of the sensitivity of spectrofluorometers and the lower stability of proteins in very dilute solutions, these measurements usually are carried out using protein concentrations that are higher than 50−100 nM. Consequently, when proteins with very high affinities for their ligands (i.e., with dissociation constants in the nanomolar range) are studied, titration curves will be obtained using protein concentrations that are significantly higher than the K_d and will reflect stoichiometric rather than equilibrium binding. Such measurements are useful for obtaining reliable estimates of the number of binding sites and of upper limits for K_ds, but may not yield accurate information about variations in dissociation constants at the low range.

To circumvent these limitations, binding of 9cRA to RXR was studied by assessing the partitioning of this ligand between RXR and unilamellar phospholipid vesicles. Because of their amphipathic nature, retinoids display a high affinity for lipid bilayers. The introduction of another phase with high affinity for the ligand results in equilibrium distribution of 9cRA between the vesicles and RXR which can be measured at concentration of the receptor in which the protein is stable. Since the affinity of retinoids for lipid bilayers is constant as long as the concentration of the ligand in the membranes is kept low (<5 mole%, Noy & Xu, 1990a,b), differences in partitioning constants of 9cRA between RXR and lipid bilayers will solely reflect variations in the affinity of the protein for the ligand.

To monitor the association of 9cRA with lipid bilayers, unilamellar vesicles of DOPC containing the fluorescent PY-PC were used. The fluorescence emission spectrum of PY-PC significantly overlaps with the absorption spectrum of 9cRA. Consequently, the presence of 9cRA in close proximity to the probe within the lipid bilayers will result in quenching of probe fluorescence. A calibration curve was constructed relating the fluorescence of PY-PC incorporated in the vesicles to the concentration of 9cRA within the bilayers. Since fluorescence values depend on the exact conditions of the experiment (lamp output, setting of slits, etc.), calibration curves were re-constructed for every experiment. A representative calibration curve is shown in Figure 2. To monitor the partitioning of 9cRA between RXR and the vesicles, vesicles were mixed with RXR and the mixture was titrated with 9cRA. The concentration of 9cRA within the bilayers at equilibrium was extracted from the change in fluorescence at each point and the calibration curve. The concentration of protein-bound ligand was calculated by subtracting the concentration of vesicle-associated 9cRA from the total concentration of added ligand. The calculation was thus based on the assumption that, owing to its low solubility in water, 9cRA in assay mixtures partitioned between vesicles and RXR. The level of saturation of RXR binding sites with 9cRA (ν) was plotted as a function of the total concentration of 9cRA (Figure 3). The data indicated that the binding affinity of RXR to 9cRA became stronger as the saturation level of the protein increased. This can be seen from the initial lag in the increase in ligand binding by RXR and the steeper increase in this parameter at total 9cRA concentrations higher than 0.15 μM. The measurements could not be extended to receptor saturation levels higher than 0.25, since the observed fluorescence values at total ligand concentrations higher than 0.4 μM were beyond the

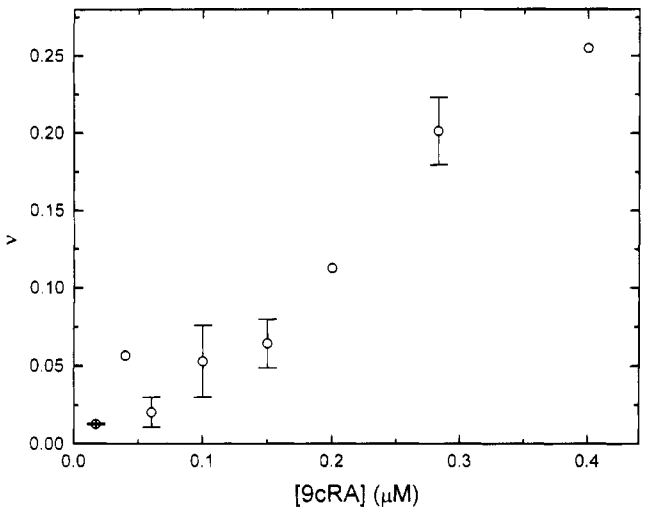

FIGURE 3: Saturation level of RXR as a function of total concentration of 9cRA. Small unilamellar vesicles of DOPC (20 μM) containing the fluorescent probe PY-PC were mixed with RXR (0.5 μM) and titrated with 9cRA. The fluorescence of the probe was measured at each titration step. The concentrations of vesicle-associated 9cRA were obtained from the relative quenching of probe fluorescence at each titration step using the calibration curve shown in Figure 2. The concentrations of protein-bound 9cRA was calculated by subtracting the concentration of 9cRA in the vesicles from the total amount of ligand added (see text). Error bars represent S.E.M. of values from three independent experiments. Points with no error bars represent the mean of two measurements.

linear range of the calibration curve, and could not be reliably used to extract the concentration of 9cRA in the vesicles. Nevertheless, the data depicted in Figure 3 indicate that binding of 9cRA to RXR is characterized by positive cooperativity.

These data suggest that the positive cooperativity in ligand-induced dissociation of RXR tetramers stems from positive cooperativity in binding of 9cRA to this receptor.

Rate of Dissociation of 9cRA from RXR Tetramers and from RXR Dimers. The observed positive cooperativity in ligand binding by RXR implies that occupation of the first ligand-binding site of receptor tetramers allosterically induces a conformational change that results in a higher ligand-binding affinity in subsequent sites (see Discussion). The data thus suggest that subunits of holo−RXR, which exists as a dimer, will display a higher affinity for 9cRA as compared to subunits that are part of the unliganded RXR tetramer. Since ligand-binding and oligomerization of RXR are intimately connected, investigation of this question by direct binding studies is precluded. Instead, the kinetics of the dissociation of 9cRA−RXR complexes was examined. The dissociation rate constant (k_{off}) is related to the equilibrium dissociation constant (K_d) by the equation: $K_d = k_{off}/k_{on}$, where k_{on} is the rate constant for association of 9cRA with RXR. Differences in K_d values therefore may reflect differences in k_{on} as well as in k_{off}. k_{on} could not be measured directly as it is too rapid, but it was of interest to clarify whether differences can be found between the rate constants for dissociation of 9cRA from RXR under conditions where the receptor exists mainly as a tetramer or mainly as a dimer, i.e., at low and at high saturation levels of the receptor, respectively.

To induce dissociation of 9cRA from RXR, the receptor was complexed with the ligand and mixed with vesicles of DOPC. Following mixing, the ligand partitioned from the protein to the vesicles and the transfer reaction was followed. Binding of 9cRA to RXR results in quenching of the intrinsic fluorescence of the protein (Chen *et al.*, 1994; Kersten *et al.*, 1995b). Movement of 9cRA from RXR to vesicles could thus be followed by monitoring the time-dependent release of quenching of protein fluorescence following mixing of the RXR−9cRA complex with vesicles. If transfer is allowed to proceed to completion, i.e., if all the ligand is transferred at equlibrium, the rate of the reverse reaction will be negligible, and the rate constant of the observed reaction will directly reflect the rate constant for dissociation of 9cRA from RXR (k_{off}) (Daniels *et al.*, 1985). Completion of the transfer reaction was verified by the observations that the rate constant was independent of the concentrations of the vesicles when lipid concentrations higher than 1 mM were used (range tested, 0.1−5.0 mM lipids, data not shown).

To determine the rate constant for the dissociation of 9cRA from tetrameric RXR, measurements were carried out at a ligand/receptor molar ratio of 0.1, a saturation level at which RXR exists predominantly as a tetramer (Figure 1). Protein preparations used in this study possessed 0.7−0.85 mol of binding sites per mole protein (see Materials and Methods). Thus, to measure the rate constant for dissociation of 9cRA from RXR dimers while avoiding the presence of excess 9cRA, holo-RXR, which is predominantly dimeric, was prepared by complexing the ligand with the receptor at a molar ratio of 0.7. Representative traces reflecting transfer of 9cRA from RXR tetramers and from RXR dimers to vesicles are shown in Figure 4 (A and B). The reactions, in both cases, followed a single first-order kinetic pattern and yielded dissociation rate constants of 0.42 ± 0.05 s^{-1} and 0.135 ± 0.003 s^{-1} (mean ± S.E.M; $n = 4$) at ligand/protein ratios of 0.1 and 0.7, respectively. The $t_{1/2}$s for dissociation of 9cRA from RXR tetramers and from RXR dimers are thus 1.65 s and 5.13 s, respectively, demonstrating that the rate of dissociation of 9cRA from RXR dimers is 3-fold slower as compared to dissociation of this ligand from RXR tetramers.

The finding that the rate of dissociation of 9cRA from RXR tetramers is significantly faster than that from RXR dimers, taken together with the data in Figures 1 and 3, provides further support to the conclusion that holo-RXR has a higher affinity for its ligand *vs.* apo-RXR, i.e., that ligand binding by this receptor is characterized by positive cooperativity.

Kinetics of Dissociation of RXR Tetramers Induced by Binding of 9cRA. We have previously suggested that modulation of the oligomeric state of RXR by 9cRA may constitute a regulatory step in the pathway by which 9cRA affects gene transcription (Kersten *et al.*, 1995b). It was therefore of interest to determine the rate by which RXR oligomers can respond to changes in the concentration of ligand, and thus to clarify the temporal sensitivity of the initial step of this transcriptional activation pathway. For this purpose, the rate constant characterizing the ligand-induced dissociation of RXR tetramers was determined. As described above, the fluorescence of fluorescein-labeled RXR decreased concomitantly with dissociation of receptor tetramers (Figure 1). Dissociation could thus be followed by monitoring the time-dependent decrease in the fluorescence of labeled RXR following addition of 9cRA. Fluorescein-labeled RXR was mixed with 9cRA at a final RXR−9cRA complex concentration of 1 μM, where RXR initially exists

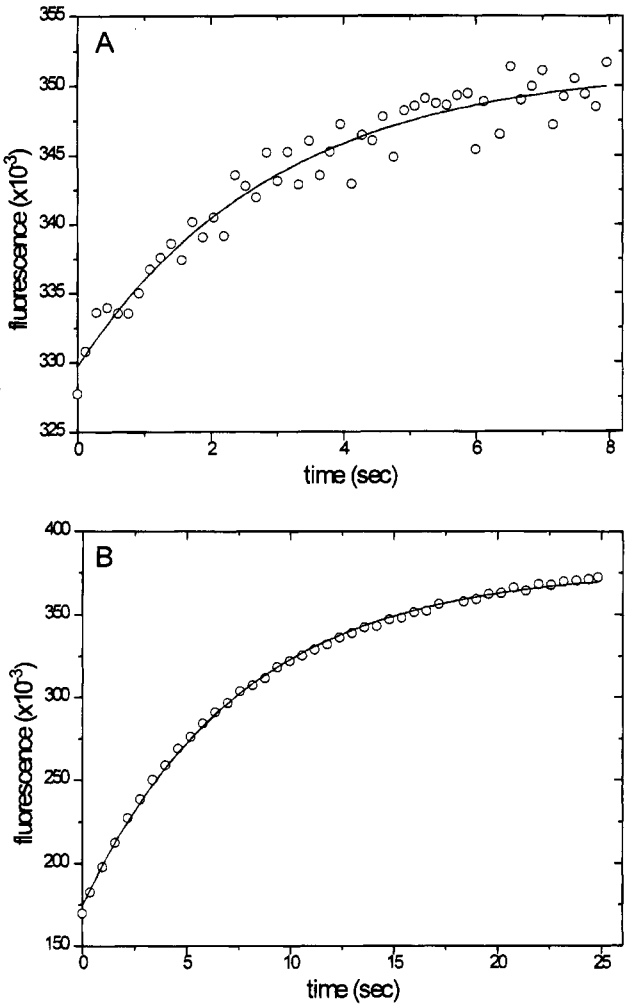

FIGURE 4: Kinetics of dissociation of 9cRA from RXR tetramers and from RXR dimers. 9cRA was complexed with RXR (2 μM) at a molar ratio of either 0.1 (A) or 0.7 (B). Complexes were mixed with small unilamellar vesicles of DOPC (2 mM) using a stopped-flow accessory. The intrinsic fluorescence of RXR (λ_{ex} = 280 nm; λ_{em} = 340 nm) was followed until equilibrium was reached. Lines represents the fit of the data according to a single first-order reaction.

as a tetramer, but after ligand binding is completely dissociated into dimers at equilibrium (see Figure 1). A representative trace of the time-dependent decrease in the fluorescence of labeled RXR upon addition of ligand is shown in Figure 5. Control experiments verified that the fluorescence of RXR did not respond to addition of all-*trans* RA (tRA), which does not bind to this receptor. The data in Figure 5 indicate that tetramer dissociation was completed within 0.6 s. Dissociation could be well described as a single first-order reaction with a rate constant of 8.6 ± 0.41 s^{-1} (mean ± S.E.M. n = 30; two independent protein preparations). Ligand-induced RXR tetramer dissociation is thus a rapid process characterized by a $t_{1/2}$ of 80 ms.

Kinetics of Dissociation of RXR Tetramers Following Dilution of the Receptor. The rapid rate of the ligand-induced dissociation of RXR tetramers raises the question of whether monomer−dimer−tetramer equilibrium is attained rapidly in the absence of ligand. It was previously determined that in the absence of ligand, the equilibrium dissociation constants for formation of RXR dimers from monomers and for formation of tetramers from dimers are 155 and 4.4 nM, respectively (Kersten *et al.*, 1995a). It can be calculated, based on these values, that tetramers comprise

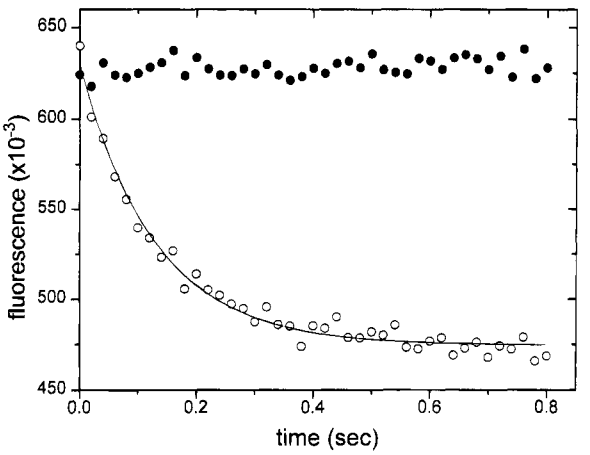

FIGURE 5: Kinetics of dissociation of RXR tetramers following binding of 9cRA. Fluorescein-labeled RXRα (2 μM) was mixed with 2 μM of either 9cRA (open circles) or tRA (closed circles). The solutions were mixed at a volume ratio of 1/1 using a stopped-flow accessory. The fluorescence of the labeled protein (λ_{ex} = 491 nm; λ_{em} = 516 nm) was monitored until equilibrium was reached. Line represents the fit of the data according to a single first-order reaction equation.

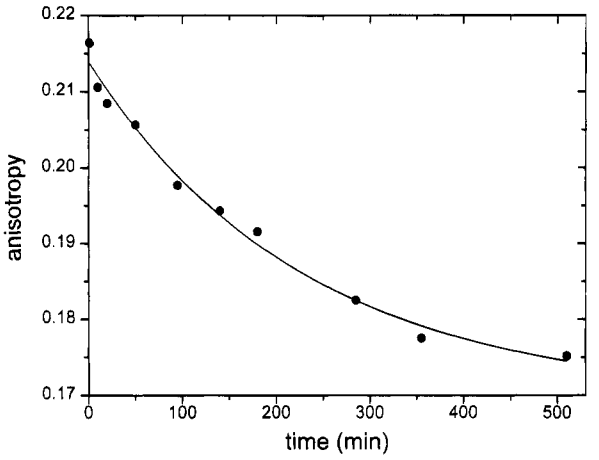

FIGURE 6: Kinetics of dissociation of RXR tetramers in the absence of ligand. Fluorescein-labeled RXRα was diluted directly into a cuvette from a concentrated solution (13 μM) to a concentration of 50 nM. The decrease in fluorescence anisotropy associated with dissociation of RXR tetramer was monitored until a constant value was approached. Line represents the fit according to a single first-order reaction equation.

the predominant RXR species at concentrations that are higher than 70 nM. Thus, to induce significant dissociation of RXR tetramers in the absence of ligand, the receptor needs to be diluted to concentrations that are lower than this value. To monitor tetramer dissociation, RXR, labeled with the fluorescent probe fluorescein, was diluted from a concentrated solution (13 μM) to a concentration at which it exists mainly as monomer and dimer (50 nM). Tetramer dissociation was monitored by following the fluorescence anisotropy of the labeled protein. The receptor was directly diluted into a cuvette, and measurements were carried out at intervals until a constant value was approached. Figure 6 shows a trace of the time-dependent change in fluorescence anisotropy following receptor dilution. The data were analyzed as a single first-order reaction to yield a dissociation rate constant of about 7 × 10^{-5} s^{-1}, or a $t_{1/2}$ of about 165 min (mean of two experiments).

These data show that dissociation of RXR tetramers is about 5 orders of magnitude slower in the absence *vs.* in the presence of 9cRA.

DISCUSSION

It was previously demonstrated that in solution, RXR forms tetramers with a high affinity (Kersten *et al.*, 1995a) and that binding of 9cRA to this receptor results in dissociation of protein tetramers to dimers (Kersten *et al.*, 1995b). Here it is shown that ligand-induced RXR tetramer dissociation is characterized by pronounced positive cooperativity. The data further suggest that this cooperativity stems from positive cooperativity in binding of ligand by RXR. This conclusion was indicated by examination of the partitioning of 9cRA between RXR and lipid vesicles which showed that the binding affinity of RXR for its ligand is weak at low saturation levels, and that it rapidly increases as higher saturation levels of the receptor are attained (Figure 3). In addition, measurements of the rate constants for dissociation of 9cRA from RXR revealed that the ligand dissociates from the receptor at a significantly slower rate when the receptor is fully saturated as compared to when the saturation level is low (Figure 4). Taken together, the data strongly suggest that ligand binding by RXR tetramers is characterized by pronounced positive cooperativity.

Cooperative behavior in ligand binding is an important regulatory property of some oligomeric proteins as it increases the sensitivity of ligand binding by a protein to small variations in ligand concentration within a certain ligand concentration range. A classic example is that of hemoglobin, a protein that exists as a tetramer and that binds oxygen in a highly cooperative fashion. In this case, positive cooperativity is important in order to enhance the sensitivity of hemoglobin to the small variations in oxygen pressure between arterial and venous blood.

A similar scenario can be envisioned regarding the role of cooperativity in ligand binding by RXR for the function of this receptor. The data in Figure 1 demonstrate that at a receptor concentration of 1 μM, the oligomeric state of RXR will be extremely sensitive to small variations in the concentration of 9cRA at the 0.3−0.6 μM concentration range. Currently, neither the concentration of RXR nor the concentration of 9cRA in the nucleus is known. It has been estimated that the concentration of one form of RAR in the nucleus is on the order of 0.5 μM (Nervi *et al.*, 1989; see Kersten *et al.*, 1995a, for discussion), which is similar to the concentration of RXR used in the experiments reported here. It is clear from the data presented above the regulation of the local concentration of the physiological ligand for RXR is an important step in modulating the process of signaling by this receptor. The enzymatic activity responsible for controlling the ligand concentration is not currently known. Attempts to identify enzymatic activities that catalyze the isomerization of all-*trans* retinoic acid to the 9-*cis* form have so far been unsuccessful (Urbach & Rando, 1994). Another possibility is that 9cRA may be generated by isomerization of 9,13, di-*cis*-retinoic acid which was identified as a relatively abundant endogenous retinoid (Horst *et al.*, 1995).

An additional consequence of the positive cooperativity in ligand binding by RXR and in the ensuing tetramer dissociation is that a threshold concentration of ligand has to be reached prior to activation. At ligand concentrations lower than this threshold, RXR oligomerization is little affected. Once this concentration is exceeded, tetramer dissociation rapidly ensues. Considering that dissociation of RXR tetramers seems to be the initial step in signaling by 9cRA, the existence of a threshold may be important in order to ensure that low, background, concentrations of this potent ligand will not result in unscheduled effects on transcription.

Positive cooperativity is often explained in terms of the so-called sequential model which states that occupation of the first ligand binding site allosterically induces a conformational change, resulting in a higher binding affinity in subsequent sites. This would imply the existence of intersubunit interactions within RXR tetramers. It also suggests that ligand binding sites can adopt at least two different conformations: one conformation that is characterized by low binding affinity, and another conformation with a high affinity for the ligand. The findings of the present work indicate that in addition to high affinity sites in RXR tetramers, subunits within holo−RXR, which exists as a dimer, are also in a high-affinity binding state. It cannot be ascertained from the data reported here whether the conformation of the high-affinity sites within dimers is the same as that of high-affinity sites within tetramers. However, since ligand binding by RXR leads both to conversion of binding sites to a high-affinity state and to dissociation of tetramers, it seems likely that the two processes are closely coupled; i.e., that conformational changes that accompany the conversion of the low to high affinity state are also responsible for decreasing the affinity of RXR dimers for each other and leading to tetramer dissociation.

The structure of the ligand binding domain of apo-RXRα was recently resolved by X-ray crystallography (Bourguet *et al.*, 1995). Though the ligand binding site could not be definitively identified in this study, two large hydrophobic pockets were revealed. Modeling experiments showed that both pockets could potentially accommodate a 9cRA molecule with minor conformational adjustments of the surrounding regions. This is surprising in view of the data presented here and in previous reports (Leid, 1994; Kersten *et al.*, 1995b) which indicated that ligand binding by RXR is accompanied by significant conformational alterations. However, as was pointed out (Bourguet *et al.*, 1995), it is possible that significant structural changes could be associated with only slight modifications in the environment within the ligand binding cavity.

Ligand-induced dissociation of RXR tetramers displayed a $t_{1/2}$ of 80 ms (Figure 5). This observation indicates that the oligomerization state of RXR very rapidly responds to changes in the concentration of ligand, and suggests that tetramer dissociation is not likely to comprise a rate-limiting step in transcriptional activation mediated by RXR. Dissociation of RXR tetramers in the absence of ligand was found to be about 5 orders of magnitude slower than dissociation in the presence of ligand (Figure 6), further emphasizing the stability of apo-RXR tetramers, and demonstrating that ligand binding by the receptor significantly lowers the energy of activation for tetramer dissociation.

RXR, in contrast with hemoglobin, has more complex functions than merely binding and releasing its ligand. Binding of ligand, in this system, functions to initiate transcriptional activation by the receptor. The mechanisms by which transactivation is initiated following ligand binding

are not well understood as yet. The data in this and in previous reports (Kersten *et al.*, 1995a,b) demonstrate that 9cRA modulates the oligomeric state of RXR, and that this process is tightly regulated by the precise concentrations of the ligand *via* a pronounced positive cooperativity in ligand binding by the receptor. These findings strongly suggest that dissociation of RXR tetramers in response to 9cRA is an important regulatory step in the pathway by which retinoids modulate gene transcription.

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REFERENCES

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., & Moras, D. (1995) *Nature 375*, 377−382.
Chen, Y., & Noy, N. (1994) *Biochemistry 33*, 10658−10665.
Chen, Z.-P., Shemshedini, L., Durand, B., Noy, N., Chambon, P., & Gronemeyer, H. (1994) *J. Biol. Chem. 269*, 25770−25776.
Cogan, U., Kopelman, M., Mokady, S., & Shinitzky, M. (1976) *Eur. J. Biochem. 65*, 71−78.
Daniels, C., Noy, N., & Zakim, D. (1985) *Biochemistry 24*, 3286−3292.
Dittmer, J. C., & Wells, M. A. (1969) *Methods Enzymol. 14*, 482−530.
Durand, B., Sauders, M., Leroy, P., Leid, M., & Chambon, P. (1992) *Cell 71*, 73−85.
Fernando, T., & Royer, C. (1992) *Biochemistry 31*, 3429−3441.
Giguère, V. (1994) *Endocrinol. Rev. 15*, 61−77.
Glass, C. K. (1994) *Endocrinol. Rev. 15*, 391−407.
Horst, R. L., Reinhardt, T. A., Goff, J. P., Nonnecke, B. J., Gambhir, V. K., Fiorella, P. D., & Napoli, J. L. (1995) *Biochemistry 34*, 1203−1209.
Kersten, S., Kelleher, D., Chambon, P., Gronemeyer, H., & Noy, N. (1995a) *Proc. Natl. Acad. Sci. U.S.A 92*, 8645−8649.
Kersten, S., Pan, L., Chambon, P., Gronemeyer, H., and Noy, N. (1995b) *Biochemistry* (submitted).
Kwon, O.-S., & Churchich, J. E. (1994) *J. Biol. Chem. 266*, 266−271.
Leid, M. (1994) *J. Biol. Chem. 269*, 14175−14181.
Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S., & Chambon, P. (1992) *Cell 68*, 377−395.
Mangelsdorf, D. J., Umesono, K., & Evans, R. M. (1994) in *The Retinoids* (Sporn, M. B., Roberts, A. B., & Goodman, D. S., Eds.) pp 319−349, Raven Press, New York.
Nervi, C., Grippo, J. F., Sherman, M. I., George, M. D., & Jetten, A. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5854−5858.
Noy, N., & Xu, Z.-J. (1990a) *Biochemistry 29*, 3883−3888.
Noy, N., & Xu, Z.-J. (1990b) *Biochemistry 29*, 3888−3892.
Noy, N., Slosberg, E., & Scarlata, S. (1992) *Biochemistry 31*, 11118−11124.
Ong, D., & Chytil, F. (1980) *Methods Enzymol. 67*, 288−296.
Urbach, J. E., & Rando, R. R. (1994) *Biochem. J. 299*, 459−465.
Yu, V., Delsert, C., Andersen, B., Holloway, J. M., Devary., O. V., Näär, A. M., Kim, S. Y., Boutin, J.-M., Glass, C. K., and Rosenfeld, M. G. (1991) *Cell 67*, 1251−1266.
Zhang, X.-K., Lenmann, J., Hoffmann, B., Dawson, M. I., Cameron, J., Graupner, G., Hermann, T., Tran, P., & Pfahl, M. (1992) *Nature 358*, 587−595.
Zhang, X.-K., Salbert, G., Lee, M.-O., & Pfahl, M. (1994) *Mol. Cell. Biol. 14*, 4311−4323.

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