The Ligand Binding Domain of the Human Retinoic Acid Receptor γ Is Predominantly α-Helical with a Trp Residue in the Ligand Binding Site*

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Retinoic acid exerts its many biological effects by interaction with a nuclear protein, the retinoic acid receptor (RAR). The details of this interaction are unknown due mainly to the lack of sufficient quantities of pure functional receptor protein for biochemical and structural studies. We have recently cloned the D and E domains of human RARγ for expression in Escherichia coli. Using nickel-chelation affinity chromatography with polyethylene amine-aminoterminal tail, purification of the DE peptide with a pl of 5.18 was accomplished to greater than 98% purity. Scatchard analysis and fluorescence quenching techniques using the purified protein indicate a very high percentage of functional molecules (>95%) with a Kd for retinoic acid (t-RA) of 0.6 ± 0.1 nM. Circular dichroism spectra of the purified domains predict a predominantly α-helical structure (~56%) with little β sheet present. No significant changes in these structural characteristics were observed upon binding of t-RA. Inspection of the amino acid sequence within these domains identified a single tryptophan residue at position 227. Modeling the amino acid sequence in this region as an α-helical structure indicates that this tryptophan is adjacent to alanine 234, which corresponds to alanine 225 in RARβ that has previously been linked to the ligand binding site. Fluorescence of this tryptophan was quenched in a dose-dependent manner on the addition of t-RA, confirming that Trp-227 is within the ligand binding site. Tryptophan fluorescence quenching analysis also demonstrates that a single retinoic acid molecule is bound per receptor and suggests that receptor-ligand interactions occur within the amino-terminal portion of the predominantly α-helical ligand binding domain.

Retinoids, derivatives of vitamin A, play important roles in morphogenesis, differentiation, and cellular proliferation (1–3). Their action, at the molecular level, is mediated by several nuclear receptors belonging to the steroid/thyroid receptor superfamily (4). Three retinoic acid receptors (RARα, RARβ, and RARγ) bind all-trans retinoic acid (t-RA) and 9-cis RA, while three retinoid X receptors (RXRα, RXRβ, and RXRγ) bind 9-cis RA but not t-RA (5–7). As a class, these receptors are ligand-inducible trans-acting transcription factors, which can modulate the expression of specific target genes by interaction with cis-acting DNA sequences termed retinoic acid response elements (8–10). The RARs, like other members of this nuclear receptor superfamily, have a modular structure consisting of six domains denoted A through F (11–13). The E region, or ligand binding domain, is 85–90% conserved among the RARs and has ligand-dependent transactivation and dimerization functions (14–16).

Information pertaining to the interaction between the RARs and their ligand requires large quantities of purified receptor protein in a functional three-dimensional conformation. Several attempts have been made at purification of recombinant full-length RARα, RARβ, and RARγ expressed in eukaryotic cells (6, 17, 19), Sf9 insect cells using the baculovirus expression system (18, 19), and a variety of expression systems in Escherichia coli (20, 21). Expression of subfragments corresponding to the RAR ligand binding domain (i.e. domains DEF, EF, etc.) in E. coli improved yields as compared to expression of full-length receptors (17, 22, 23). However, in every case, regardless of yield, the purified receptor preparations were insufficiently active with respect to ligand binding and were therefore unsuitable for structural or biochemical analysis.

Improved expression and purification techniques described here using nickel chelation chromatography and a modified thrombin cleavage produced milligram quantities of hRARγ receptor protein containing the D and E domains. This DEγ protein was found to have binding kinetics similar to that of the native full-length receptor. Circular dichroism analysis of the expressed protein suggests that the secondary structure of the ligand binding domain is predominantly α-helical and contains very little β sheet. This observation is in good agreement with the reported crystal structure of the ligand binding domain of RARγ, a closely related member of the steroid/thyroid hormone receptor superfamily (24), and that determined from structure prediction modeling. Interestingly, the relative pattern of secondary structural components for this peptide did not change significantly upon binding to ligand.

Inspection of the amino acid sequence within the D and E domains revealed a single tryptophan residue at position 227, which was subsequently used for fluorimetric titration. At a concentration of protein determined by amino acid analysis, fluorescence was quenched in a dose-dependent manner upon addition of t-RA with a transition point equal to the concentration of protein in the reaction. Coganan analysis (33) of this curve indicates a single ligand binding site within DEγ. Further, the position of the transition point indicates that ~95% of the receptor protein is present in an active conformation and that...
Trp-227 is located in close proximity to the ligand binding site. The assignment of Trp-227 within the ligand binding site is further supported by a previous report that Ala-224 (Ala-225 in RARβ) is a contact amino acid within the ligand binding site (25). In an α-helical structure, this alanine would be expected to be immediately adjacent to Trp-227 supporting a structural model for the ligand binding pocket.

**EXPERIMENTAL PROCEDURES**

**Materials—**Expression vector pET15b, host strain E. coli BL21(DE3), restriction grade thrombin and His-bindTM resin were purchased from Novagen, Inc. pSG5/hRARα was kindly provided by Dr. P. Chambon (26). Tag polymerase, PCR buffer (annealing, 100% ethanol), and deoxyribonucleotides were purchased from Perkin-Elmer Corp. Amplification was performed in an Ercocomp EasyCycler™ (San Diego). Ligand binding assays were carried out with t-RA, retinol, and retinol purchased from Sigma and [11,12-3H]-t-RA (47.5 Ci/mM) from DuPont NEN. 9-cis RA was synthesized by Bristol-Myers Squibb Central Chemistry (Wallington, CT). Retinoids were used under yellow fluorescent light to minimize photodegradation. Oligonucleotides used in the PCR reactions were synthesized by Genosys Biotechnologies, Inc. (Woodlands, TX). Restriction enzymes and DNA ligase were purchased from New England Biolabs, Inc. All other chemicals used were of reagent grade and purchased from Sigma.

**Construction of pET15b/DE γ Expression Vector—**The truncated DE γ receptor cDNA was constructed and amplified from the full-length hRARα cDNA (26) using PCR with the upstream forward primer 1 (36-mer) 5'-CTCGCATAGACCCTATGGCAAGAAGCTTGGCA-3' and the downstream reverse primer 2 (35-mer) 5'-GGCCGGGATCC(TTA)CATTCTGGAACGTTGACTGAAC-3'. The underlined nucleotides in primer 2 represent the hybridizing portion, and the nucleotides in boldface type specify the Ndel and BamHI restriction sites, respectively. The codon specifying the translation stop is in parentheses in primer 2. After 5 min of preheating at 95 °C, each of 20 PCR cycles was as follows: denaturation, 1 min at 94 °C; annealing, 1 min at 55 °C; and synthesis, 2 min at 72 °C. The 50-μl PCR mixture contained 50 pmol of each primer, 3 ng of pSG5/hRARα, 250 μM dNTPs, 50 μM each of Ndel and BamHI restriction enzymes, 10 μM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 0.001% gelatin, and 1.0 unit of Taq polymerase. Following amplification, the reaction was extracted with an equal volume of chloroform/isooctane (24:1) and then precipitated and washed with 100% ethanol. The resultant cDNA was digested to completion with Ndel and BamHI and gel purified and ligated into the similarly digested recipient pET15b vector. The ligation mixture was used to transform competent E. coli strain DH5α cells. The sequence of the ligated insert from a transformed colony was confirmed using a modification of the Sanger dideoxy method (U. S. Biochemical Corp.) and was designated pET15b/DE γ.

**Expression of DE γ in E. coli and Preparation of Crude Soluble Extract—**For the preparation of purified fusion proteins, 12 × 108 BL21(DE3)pET15b/DE γ cells were introduced into 1 liter of minimal medium (MM/C) (42 mM Na2HPO4-7H2O, 22 mM KH2PO4, 8.6 mM NaCl, 19 mM NH4Cl, 5 μg/ml FeCl3·6H2O, 1 mM MgSO4·7H2O, 100 μM CaCl2, 0.0001% thiamine, 0.4% glucose, 50 μg/ml carbenicillin) prewarmed to 37 °C. The inoculum was freshly prepared in MM/C from a frozen stock of the expression host BL21(DE3)/pET15b-DE γ. The cells were grown at 37 °C in a New Brunswick model G25 shaker at 350 rpm. At 1.0 A600 (approximately 18 h), the temperature of the culture was rapidly shifted to 26 °C by swirling on ice water. After 3 h at 26 °C with continuous shaking at 350 rpm, the cells were harvested by centrifugation at 4,200 × g for 5 min at 4 °C, resuspended in 50 ml of ice-cold 50 mM Tris-HCl, pH 8.0. After addition of lysozyme and Triton X-100 to final concentrations of 200 μg/ml and 0.1%, respectively, the cells were quickly frozen on dry ice, thawed at 30 °C, and then sonicated for 30 s until no longer viscous. After the addition of NaCl to 0.5 ×, the lysate was centrifuged at 15,000 × g for 30 min at room temperature, the supernatant was purified by nickel affinity chromatography. The DE γ protein was purified from 150-200 ml of crude soluble cytoplasmic extract in a batchwise fashion by mixing with a 5-ml bed volume of Ni2+-charged His-Bind resin equilibrated in binding buffer (50 mM imidazole, 20 mM Tris-HCl, pH 7.9). The resin was washed with Novagen and prepared according to the manufacturer’s instructions. After gently rotating 45–60 min at room temperature, the resin was packed into a 2.5 × 100-cm Econocolum (Bio-Rad) and washed twice with resuspension in 50 ml of binding buffer, followed by four washes in 50 ml of wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). When the concentration of protein in the final wash was less than 100 μg/ml, the resin was equilibrated with cleavage buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2.5 mM CaCl2) and the amount of protein bound to the resin was determined by the Bradford method (27). Thrombin (Novagen) was added to a final concentration of 0.5 units/mg protein in a volume of 20–30 ml of cleavage buffer and allowed to cleave for 12–14 h at 25 °C while rotating slowly in a vessel with no discernible airspace. The column was drained and then rinsed with cleavage buffer without CaCl2. Phenylmethylsulfonyl fluoride was added to the eluate from a slow to a fast concentration of 11 mM to inhibit thrombin activity. The purity of the DE γ protein was determined by laser densitometry of silver-stained 12% SDS-polyacrylamide gel electrophoresis gels (28) and by amino acid analysis.2

**Isoelectric Focusing—**The isoelectric point of the purified DE γ protein was determined using the Pharmacia Phast System. Pharmacia broad pl calibration standards were run in parallel lanes with DE γ on a Phast Gel IEF 3–9. The gel was prefocused at 2000 volts, 2.5 mA, 3.5 watts for 75 V-h at 15 °C. After loading and prerunning the gel at 200 V, 2.5 mA, 3.5 watts for 15 V-h at 15 °C, the samples were focused for 410 V-h under the same conditions as in the prefocusing step. The gel was silver stained according to the manufacturer’s instructions and dried under hot air for 10 min. Migration distances for each of the standards were measured and marked versus isoelectric points. The pl for the DE γ protein was determined by comparison with this standard curve.

**Ligand Binding Assays—**In binding assays and competition experiments, 1.0–3.0 pmol of purified DE γ receptor protein plus 3.5 μg of uninduced crude extract, which served as a carrier and had negligible specific binding of its own, was routinely added to binding buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl) giving a final volume of 1.0 ml. In the binding assays, the protein was incubated with 0.1–10.0 nM [3H]-t-RA. For competition experiments, the protein mixture was preincubated with 1.0 nM [3H]-t-RA at 4 °C for 30 min, after which various concentrations of unlabeled retinoids solubilized in EO/THF were added. The final concentration of EO/THF did not exceed 1–2% (v/v). The reactions were carried out in the dark at 4 °C for 1.5 h, after which 0.5 ml of ice-cold equivalent particle size, lyophilized dextran-coated charcoal (EDC/DCC), prepared according to Dokoh et al. (29), was added. The samples were vortexed, placed on ice for 10 min, and then centrifuged 15 min at 14,000 × g at 4 °C. Total dpm of the supernatants was measured in a Beckman LS6500 IC scintillation counter. Nonspecific binding was determined in the presence of 100-fold molar excess unlabeled t-RA. Scatchard analysis (30) was performed for determination of Kp. Apparent Kp values for t-RA, 9-cis RA, retinol, and retinyl acetate were calculated using the ICKp determined from competition curves and the Clark equation (31).

**Circular Dichroism Measurements—**CD spectra were recorded with a Jasco 710 spectropolarimeter (Jasco Inc., Tokyo). The instrument was calibrated at ambient temperature such that a 1 mg/ml solution of (+)−10-camphor sulfonic acid had an ellipticity ratio of 2.0 when measured at 192.5 and 290 nm in a cell with a 0.1-mm optical path length. All experimental measurements were made at ambient room temperature (22.2 ± 0.1 °C) on samples of 50 μM protein in buffer containing 50 mM Tris (pH 7.4), 100 mM NaCl, 0.1 mM EDTA, and 0.025 mM phenylmethylsulfonyl fluoride. In some samples, t-RA was added in equimolar amounts to protein. CD spectra were recorded using buffer alone as the baseline. For each sample, four spectra were collected and averaged (two spectra for two separate preparations of each sample). All spectra are reported in terms of molar ellipticity, [θ], where [θ] = [θ]x/[θ]0 × 100, and θ is the observed mean residue ellipticity, L is the path length, and c is the protein concentration. CD spectra were deconvoluted by least squares analysis with the four-basis set of Yang and co-workers (32). Estimates of secondary structure were obtained by normalizing the fitted parameters to yield percentages of helix, sheet, turn, or random coil.

**Fluorescence Measurements—**Fluorescence measurements were made with a Perkin-Elmer model LS-5B (Perkin-Elmer Corp.) luminescence spectrometer at 25 °C using a slit width of 5 nm. t-RA was added to a 5 μM solution of purified DE γ protein in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl. The protein concentration was determined by amino acid analysis.2 The final concentration of ETOH in each reaction mixture did not exceed 1–2% (v/v).

2 Amino acid analysis of the purified DE γ protein was done in the Harvard Microchemistry Facility of Dr. William S. Lane.
not exceed 2% (v/v). Protein quenching was monitored at 280 nm excitation and 340 nm emission. Calculation of n (number of binding sites) was performed by the method of Cogan et al. (33). Briefly, a plot of F0/F vs. Rn/(1 - Rn) was made, where F0 = protein concentration and Rn = retinoid concentration. α is determined by (F0 - Fmax)/(F0 - F0) where F0 = initial fluorescence, F = fluorescence at each Rn, and Fmax = fluorescence at maximum quench. The resulting straight line has a slope of 1/n.

For denaturation studies, fluorescence measurements were obtained by diluting the purified DEγ protein in 6 M guanidine HCl, and emission spectra were obtained as described above in the absence of t-RA.

Results—The structural predictions for the DEγ protein were performed using the Gene-Works 2.4 DNA-protein analysis software (IntelliGenetics, Inc.).

RESULTS

Expression and Purification of hRARγ DE Domain from E. coli—The host E. coli strain, BL21(DE3), transformed with the vector pET15b/DEγ, produces a protein of the predicted molecular mass for HIS6-DEγ (approximately 32.4 kDa) upon induction with 1 mM isopropyl-β-D-thiogalactopyranoside (Fig. 1, lane 1). This protein was not detectable in uninduced cells, nor was it detected in cells transformed with the parent vector alone, pET15b (data not shown). About 90% of the HIS6-DEγ expressed was present in the soluble fraction when induced as described. The remainder was recovered in the insoluble pellet formed by centrifugation of the lysate at 15,000 × g. Induction in minimal media at 26 °C for 2 h was critical to the expression of HIS6-DEγ in the soluble fraction. Induction at 30 °C for 2 h reduced the level of soluble fusion protein by 50%, while induction at 37 °C for 2 h reduced the level beyond detection. The hydrophilic D domain appears to be important for the expression of soluble protein, since similar constructs of the E domain, which did not contain the D domain, were expressed entirely as insoluble inclusion bodies (data not shown).

The HIS6-DEγ peptide was purified from the crude soluble cytoplasmic extract by nickel chelation chromatography. The yield was approximately 7 mg of DEγ from 383 mg of crude protein (Table I) representing about 2% of the total soluble protein and is reported as the average of five experiments. The HIS6-DEγ fusion is undetectable in the void volume (Fig. 1, lane 2), indicating that the expressed fusion protein was efficiently bound to the affinity resin. Repeated washing of the affinity column with 60 volumes of a 5 mM imidazole buffer followed by 40 volumes of a 60 mM imidazole buffer was necessary to completely remove contaminating E. coli proteins from the resin.

The purified DEγ protein was eluted by thrombin cleavage of the resin-bound HIS6-DEγ fusion. Based on quantitative estimates using laser densitometry, greater than 85% of the HIS6-DEγ peptide was cleaved by thrombin. Approximately 60% of the cleaved product was recovered in the column eluate, and the purity of this protein was greater than 98% (Fig. 1, lane 5). Results from amino acid analysis of the protein in this fraction indicate that the composition of residues in the purified DEγ protein is consistent with that determined from the primary sequence so that the protein preparation is unlikely to be contaminated. Subsequent treatment of the column with buffer containing 400 mM imidazole elutes the remaining DEγ protein (Fig. 1, lane 6). Since the DEγ in this fraction does not bind retinoic acid (data not shown) and spontaneously precipitates upon collection, it most likely contains molecules that are incorrectly folded.

The isoelectric point (pI) of the purified DEγ protein was determined on a Pharmacia IEF 3–9 Phast Gel and silver stained. Isoelectric points of the standards are plotted against their migration distance from the cathode (pl points are listed in parentheses): lentil lectin basic (6.85), lentil lectin middle (6.45), lentil lectin acidic (6.15), horse myoglobin acidic (6.85), human carbonic anhydrase B (6.55), bovine carbonic anhydrase B (5.85), β-lactoglobulin A (5.20), soybean trypsin inhibitor (4.55), and amyloglucosidase (3.50). The open square indicates the position of purified DEγ protein.

![Fig. 1. SDS-polyacrylamide gel electrophoresis analysis of DEγ purification.](http://www.jbc.org/)

**Fig. 2. Determination of the isoelectric point of human E. coli-derived DEγ protein.** Broad pl calibration standards were run on a Pharmacia IEF 3–9 Phast Gel and silver stained. Isoelectric points of the standards are plotted against their migration distance from the cathode (pl points are listed in parentheses): lentil lectin basic (6.85), lentil lectin middle (6.45), lentil lectin acidic (6.15), horse myoglobin acidic (6.85), human carbonic anhydrase B (6.55), bovine carbonic anhydrase B (5.85), β-lactoglobulin A (5.20), soybean trypsin inhibitor (4.55), and amyloglucosidase (3.50). The open square indicates the position of purified DEγ protein.
Fig. 3. Saturation binding and Scatchard analysis. a, 1.0 pmol of purified DEγ was incubated with the indicated concentrations of [3H]-t-RA in the presence or absence of a 100-fold molar excess of unlabeled retinoic acid as described under “Experimental Procedures.” ○, total binding activity; ●, specific binding activity; △, nonspecific binding activity. b, Scatchard plot of the binding data. The calculated Kd was 0.60 nM (±0.1), where n = 3.

The tryptophan fluorescence emission spectrum was used to evaluate the integrity of the three-dimensional structure of the folded DEγ protein. The fluorescence of the native protein exhibits two maxima at 320 and 340 nm (Fig. 5). Upon denaturation with 6 M guanidine HCl, Trp-227 fluorescence shifts to higher wavelengths (340–350 nm), indicating increased exposure of that residue to the solvent. This shift is most likely the result of a repositioning of the tryptophan residue into a more polar, less shielded environment and is an indication that the purified DEγ protein was properly folded prior to denaturation. At the same time, relative fluorescence maxima of both the native and denatured protein remained the same (68 versus 75%), consistent with the view that fluorescence quenching was not the result of changes in conformation.

Trp-227 fluorescence can also be used to monitor binding kinetics of the purified receptor protein. Titration of 5 μM DEγ protein with increasing concentrations of t-RA reveals significant quenching of the 340-nm fluorescence peak (Fig. 6a). Since the concentration of t-RA at the inflection point of the curve (5.1 μM) is very close to the concentration of receptor protein in each reaction vessel (5.0 μM determined by amino acid analysis),2 greater than 95% of receptor molecules are folded into a functionally active conformation. Further, linearization of the fluorescence quench data by the method of Cogan (33) (Fig. 6b) was used to demonstrate the presence of one ligand binding site per receptor molecule (n = 1.08) from the slope of the Cogan curve.

**DISCUSSION**

Information concerning the three-dimensional structure of members of the steroid/thyroid hormone receptor superfamily has been hindered by the inability to purify sufficient quantities of receptor protein for NMR or x-ray crystallographic analysis. The availability of DEγ protein, from which we have constructed an alanine scan that shows the presence or absence of a 100-fold molar excess of unlabeled retinoic acid as described under “Experimental Procedures.” ○, total binding activity; ●, specific binding activity; △, nonspecific binding activity. b, Scatchard plot of the binding data. The calculated Kd was 0.60 nM (±0.1), where n = 3.

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**DISCUSSION**

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**hRARγ Domain E Contains an α-Helical Ligand Binding Site**

**Fig. 4. Structural analysis of purified DEγ protein expressed in E. coli.** A, secondary structure prediction of amino acids 155–421 of DEγ after Garnier et al. (34). B, CD spectra (plotted as the molar ellipticity, [θ], versus wavelength) are shown for DEγ protein in the absence (line b) and presence (line a) of 1 μM t-RA.

Several laboratories have reported on the use of expression systems in different species as a method for increasing yields of heterologous protein, but the yields of purified protein with functional activity have generally been low (6, 17–23). Another approach has been to express each domain separately under the assumption that a three-dimensional structure obtained in this way would be identical to that of the same domain in the context of the full-length receptor protein. The most successful use of this approach has been the expression domain in the context of the full-length receptor protein. The method described here consistently produced 5–8 mg of purified DEγ protein from 1 liter of bacterial culture, representing approximately 30% recovery of expressed protein (Table I). Purity and integrity of protein obtained in this way was assessed by a number of criteria. Silver-stained polyacrylamide gel electrophoresis gave a single band of the expected molecular mass, 31,000 daltons (Fig. 1, lane 5). The experimentally determined pI of the purified DEγ protein (Fig. 2) agrees with that calculated from the amino acid sequence (5.2 versus 5.18). Binding and Scatchard analysis of the purified protein gave a Kd of 0.6 nM (Fig. 3). This value is consistent with that obtained using crude protein extracts (7). In addition, competition binding assays using other naturally occurring retinoids (Table II) gave apparent Kd values similar to those published for the full-length receptor (17, 20) with the same order of affinity: t-RA > 9-cis RA > retinol > retinal.

Since the DEγ protein appeared to be purified to homogeneity by the methods described using the above criteria, it was appropriate to evaluate the secondary structure characteristics using circular dichroism. Initial analysis revealed that light scattering of the sample hindered interpretation of the CD spectra at wavelengths below 190 nm. Previous studies have shown that truncating the CD spectrum at 190 nm results in poor estimates for the amount of β sheet and turn conformations found in the protein (44, 45). However, the same studies found that because the CD for an α-helix dominates the spectrum, the analysis for α-helix is reliable regardless of the wavelength range. Given these considerations, the purified DEγ protein was found to contain a high degree of α-helical structure. Constrained deconvolution of the CD spectra to quantitate helix, sheet, or random coil conformations suggests an α-helical content as high as 56%, in good agreement with a value of 62% α-helical content determined using the Garnier algorithm (Fig. 4A). Further support for this observation comes from the recently published crystal structure of a closely related member of the steroid/thyroid superfamily, RXRα (24). This structure contains approximately 65% α-helix with only 4% β sheet.

On addition of retinoic acid, only minor changes in helical content were observed (Fig. 4B), indicating that major alterations in the secondary structure of DEγ were unlikely to occur upon ligand binding. Recently, CD analysis of the thyroid hormone receptor, another member of the steroid/thyroid hormone receptor superfamily, indicated a high proportion of α-helical structure (46). Interestingly, addition of thyroid hormone produced only minor changes in the CD spectra obtained, in agreement with the results reported here.

The finding of significant helical content for DEγ is noteworthy in light of the fact that the structure of another protein known to bind retinoids, retinol-binding protein, is primarily β
sheet. Retinol binding protein is a member of a family of proteins whose architecture consists of a β-barrel formed by two orthogonal β-sheets and four turns of α-helix at its carboxyl terminus (47, 48). Based on the results of this study, DEγ is structurally distinct from the retinol binding protein family. In support of this observation, one-dimensional NMR spectra of DEγ did not show any of the typical markers for β-sheet conformation.3 Few α-proton resonances are observed down field of water, and there is little chemical shift dispersion among the amide proton resonances that are typical of proteins with significant β-sheet or turn conformations.

Inspection of the amino acid sequence within the DEγ protein revealed a tryptophan residue at position 227. This residue was used to evaluate the native structure of DEγ as well as the kinetics of ligand binding. Denaturation of the protein with 6 M guanidine causes a shift in the emission maxima from 340 to 350 nm (Fig. 5). This shift suggests a repositioning of Trp-227 into a more polar environment (41). Since the relative fluorescence does not change, this shift is most likely due to structural differences common upon denaturation but not a change in autofluorescence. Thus, any quenching observed upon the addition of ligand will be due to ligand binding only, further supporting the position of Trp-227 within the ligand binding site.

Upon addition of increasing t-RA concentration, the stoichiometry of ligand binding to purified DEγ protein can be measured. This method has been used to evaluate the binding of retinoic acid and other naturally occurring retinoids to the cytoplasmic retinoic acid binding proteins (41, 42). Fig. 6a shows that the fluorescence of Trp-227 is quenched in a dose-dependent manner. An inflection in this binding curve can be expected at the concentration of ligand, which saturates all the binding sites present within the protein tested. As can be seen in Fig. 6a, fluorescence is progressively quenched upon addition of increasing amounts of t-RA. The inflection point of this titration curve occurs at a point slightly greater than 5 μM t-RA. Since the concentration of DEγ protein in each reaction vessel was adjusted to 5 μM based on amino acid analysis, it is evident that stoichiometric binding of a single binding site occurs for greater than 95% of the purified protein molecules. Linearization of the fluorimetric titration data for DEγ using the method of Cogan et al. (33) confirms only one t-RA binding site per receptor molecule (n = 1.08) (Fig. 6b). This result appears to be a reliable estimate of the number of binding sites (n) since this method is most accurate when high affinity binding occurs, as is the case for DEγ (43).

The results presented here predict that the ligand binding pocket of RARγ will be composed of at least one α-helical structure containing the tryptophan at position 227. A model of this region (Fig. 7) shows the location of Trp-227. Interestingly, this residue is immediately adjacent to an alanine residue at position 232. This residue is analogous to Ala-225 in RARβ, which has been recently demonstrated to be an important recognition residue for receptor-selective retinoids (25). Maksymowycz et al. (49) have predicted a similar helix-turn-helix motif in this region for all members of the steroid/thyroid hormone receptor superfamily. Taken together, these results...
suggest that the region in the amino-terminal portion of the ligand binding domain of the RARs contains the ligand recognition site and that all the members of this superfamily are likely to contain receptor-ligand contact points in this region. In addition, modeling the position of 9-cis RA within the ligand binding domain of another member of this superfamily, RXRa, from the crystal structure (24) predicts receptor-ligand contacts within the hydrophobic portion of the homologous helix that is described here for RARγ, further supporting this region as the ligand binding site.

In summary, this work describes an efficient, one-step method for the production and purification of milligram quantities of human RARγ DE domains expressed as heterologous protein in E. coli. The purified DEγ receptor protein is of high purity and significant biological activity. The protein exhibits binding affinities consistent with the full-length receptor for t-RA and other naturally occurring ligands. Secondary structural analysis using circular dichroism is consistent with a predominantly α-helical polypeptide with only minor alterations in secondary structure evident upon ligand binding. Fluorescence quenching techniques were used to identify a single hydrophobic, α-helical site within the amino-terminal portion of the ligand binding domain, which contains a tryptophan residue in the ligand binding pocket. Future studies will assess the structural and biochemical properties of this binding site.

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