Nuclear Magnetic Resonance Studies of 6-Fluorotryptophan-substituted Rat Cellular Retinol-binding Protein II Produced in Escherichia coli

ANALYSIS OF THE APOPROTEIN AND THE HOLOPROTEIN CONTAINING BOUND ALL-TRANS-RETINOL AND ALL-TRANS-RETINAL*

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Ellen Liță, Shi-jun Qian, Leslie Nader, Nien-chu Yang, Andre d'Avignon, James C. Sacchettini, and Jeffrey I. Gordon

From the Departments of Medicine, Biochemistry and Molecular Biophysics and Chemistry, Washington University, St. Louis, Missouri 63110 and the Department of Chemistry, University of Chicago, Chicago, Illinois 60637

Rat cellular retinol-binding protein II (CRBP II) is a 15.6-kDa intestinal protein which binds all-trans-retinol and all-trans-retinal but not all-trans-retinoic acid. We have previously analyzed the interaction of Escherichia coli-derived rat apoCRBP II with several retinoids using fluorescence spectroscopic techniques. Interpretation of these experiments is complicated, because the protein has 4 tryptophan residues. To further investigate ligand-protein interactions, we have utilized \(^{19}\)F nuclear magnetic resonance (NMR) spectroscopy of CRBP II labeled at its 4 tryptophan residues with 6-fluorotryptophan. Efficient incorporation of 6-fluorotryptophan (93\%) was achieved by growing a tryptophan auxotroph of E. coli harboring a prokaryotic expression vector with a full-length rat CRBP II cDNA on defined medium supplemented with the analog. Comparison of the \(^{19}\)F NMR spectra of 6-fluorotryptophan-substituted CRBP II with and without bound all-trans-retinol revealed that resonances corresponding to 2 tryptophan residues (designated \(W_{A}\) and \(W_{B}\)) undergo large downfield changes in chemical shifts (2.0 and 0.5 ppm, respectively) associated with ligand binding. In contrast, \(^{19}\)F resonances corresponding to other tryptophan residues \((W_{C}\) and \(W_{D}\)) undergo only minor perturbations in chemical shifts. The \(^{19}\)F NMR spectra of 6-fluorotryptophan-substituted CRBP II complexed with all-trans-retinal and all-trans-retinol were very similar, suggesting that the interactions of these two ligands with the protein are similar. Molecular model building, based on the crystalline structures of two homologous proteins was used to predict the positions of the 4 tryptophan residues of CRBP II and to make tentative resonance assignments. The fact that ligand binding produced residue-specific changes in the chemical shifts of resonances in CRBP II suggests that NMR analysis of isotopically labeled retinoid-binding proteins expressed in E. coli will provide an alternate, albeit it complementary, approach to fluorescence spectroscopy for examining the structural consequences of their association with ligand.

Rat cellular retinol-binding protein II (CRBP II)\(^{1}\) is a 134-amino acid 15.6-kDa intracellular protein which binds all-trans-retinol and all-trans-retinal (Li et al., 1986; MacDonald and Ong, 1987; Levin et al., 1988). In adult rats, expression of the CRBP II gene is essentially limited to the polarized columnar epithelial cells (enterocytes) located on small intestinal villi (Crow and Ong, 1986; Li et al., 1986), suggesting that CRBP II participates in the intestinal uptake and metabolism of vitamin A. Detailed analysis of the interaction of CRBP II with retinoids has been hampered by difficulties in obtaining the protein free of its bound ligand. To circumvent this obstacle, we have recently inserted the coding region of the CRBP II gene into a plasmid which directs efficient synthesis of foreign gene products in E. coli (Li et al., 1987). The purified E. coli-derived protein contains no bound retinoids. When complexed with all-trans-retinol, the "recombinant" protein has UV absorption and fluorescence spectroscopic properties that are indistinguishable from that of CRBP II isolated from rat intestine (Li et al., 1987). The fluorescence excitation spectrum shows a peak at 290 nm when monitoring emission at 480 nm (retinol fluorescence). When saturated with all-trans-retinol, 90% of the intrinsic protein fluorescence (tryptophan) is quenched. These spectral characteristics most likely represent the results of energy transfer between its tryptophan residues and the bound retinol.

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\(\dagger\) Lucille P. Marky Scholar. To whom correspondence should be addressed: Dept. of Medicine, Washington University School of Medicine, 660 S. Euclid Ave., Box 8051, St. Louis, MO 63110. Tel.: 314-362-9293.

\(\dagger\) Recipient of an American Heart Association Postdoctoral Fellowship.

\(\ddagger\) Established Investigator of the American Heart Association.

\(\dagger\dagger\) Recipient of an American Heart Association Postdoctoral Fellowship.

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**19F NMR of CRBP II Expressed in E. coli**

**Fig. 1.** Titration of 6-F-Trp-labeled and -unlabeled CRBP II with retinol. Panel A, retinol fluorescence was monitored to 490 nm with excitation at 348 nm. The results shown were obtained using a 2 μM solution of CRBP II. Panel B, protein fluorescence was monitored at 340 nm with excitation at 290 nm. O, 6-F-Trp-labeled CRBP II; X, unlabeled CRBP II. The 100% fluorescence intensities obtained with retinol fluorescence for 6-F-Trp-labeled CRBP II and unlabeled CRBP II were 38.5 and 40 units, respectively.

**Fig. 2.** Fluorescence excitation spectrum. 6-F-Trp-labeled CRBP II (---) and unlabeled CRBP II (----) were saturated with all-trans-retinol and spectra obtained by monitoring emission at 490 nm (3-nm slit width). The excitation slit width was set at 1.9 nm. Temperature = 22 °C. The arrow points to a shoulder seen in the peak of the fluorescence excitation spectrum of unlabeled holo-CRBP II which is discussed in the text.

relative contribution of each residue to the total protein fluorescence, since energy transfer may also occur between residues.

MacDonald and Ong (1987) have proposed that one of the functions of CRBP II was to accept retinal after it is generated from intestinal cleavage of β-carotene. E. coli derived apo-CRBP II also forms high affinity complexes with all-trans-retinal (K_a ~10^-7 M). All-trans-retinal can displace all-trans-retinol bound to intestinal and E. coli-derived CRBP II (MacDonald and Ong, 1987; Levin et al., 1988). Vibronic fine structure was observed in the CRBP II-retinol spectrum but not in the CRBP II-retinal spectrum. This lack of vibronic fine structure led MacDonald and Ong (1987) to propose that retinal did not interact with CRBP II in the same manner as retinol. However, conjugation of a carbonyl group in retinal will contribute an n,π* transition in the absorption characteristics of the polyenic system. Thus, qualitative differences in the contour of the absorption spectra of CRBP II-retinal and CRBP II-retinol do not constitute definitive proof that the binding interactions of these two ligands differ.

We have now developed an alternative approach to fluorescence spectroscopy for examining CRBP II-retinoid interactions, one based on nuclear magnetic spectroscopy. To investigate the structural consequences of retinoid binding to CRBP II, we first focused on its 4 tryptophan residues. By expressing CRBP II in a tryptophan auxotroph of E. coli, large amounts of the protein could be produced that had been highly substituted with the nuclear spin label 19F attached to the 6 position of the tryptophan ring. We chose 19F as the spin label because of its high sensitivity and large range of chemical shifts. In this paper we report our initial fluorescence and NMR spectroscopic characterization of 6-fluorotryptophan-labeled apo-CRBP II, CRBP II complexed with all-trans-retinol and CRBP II complexed with all-trans-retinal.

**EXPERIMENTAL PROCEDURES**

**Materials**—6-Fluorotryptophan and deuterium oxide (D_2O) were purchased from Sigma. All-trans-retinol and all-trans-retinal were obtained from Kodak (Rochester, NY). The plasmid expression vector pMON-CRP II and the bacterial strains used for producing unlabeled rat CRBP II have been described previously (Li et al., 1987). The E. coli tryptophan auxotroph W3110 trpA33 (Drapeau et al., 1968) was a generous gift of Dr. Charles Yanofsky (Stanford University).

**Production of 6-Fluorotryptophan-labeled Rat Apo-CRBP II** (Apo-6-F-Trp-CRBP II)—E. coli strain W3110 trpA33 was transformed with pMON-CRP II DNA (Li et al., 1987). An overnight culture of cells
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**FIG. 4.** Variable temperature study of 1 mM 6-F-Trp-holo-CRBP II. The spectra were obtained at temperatures ranging from 4 to 60 °C. 230-500 transients were obtained for each spectrum. A line broadening of 4 Hz was applied. Chemical shifts are referenced to the 19F signal for trifluoroacetic acid (TFA). The shoulder noted in resonance D was not observed initially with this sample and may represent minor decomposition of the preparation.

**FIG. 5.** 470.3-MHz 19F NMR spectra of 0.5 mM holo-6-F-Trp-CRBP II with a Met+ CRBP II:Met- CRBP II ratio of 45:55. Top panel, spectrum obtained at 22 °C, 800 transients. A line broadening of 4 Hz was applied. Chemical shifts are referenced to the signal for trifluoroacetic acid (TFA). A plus denotes resonances assigned to Met+ CRBP II, whereas a minus specifies resonances assigned to Met- CRBP II (see text).

protein sequencing—Automated sequential Edman degradation of purified CRBP II preparations was performed using an Applied Biosystems Model 490A gas phase sequencer and the manufacturer’s NOVAC program.

**Protein Quantitation**—All calculations of CRBP II concentration were based on quantitative amino acid analysis (Li et al., 1987). The cm for apo-6-F-Trp-labeled CRBP II was found to be 21,000 M⁻¹ cm⁻¹.

**Fluorescence Measurements**—Steady state fluorescence measurements were done with a Perkin-Elmer MFP-66 or a Spex spectrophotometer equipped with corrected spectra units and constant temperature cell holders (set at 22 °C). Retinol binding studies were performed by fluorometric titration as described previously (Li et al., 1987).

**Preparation of NMR Samples**—The concentration of purified CRBP II was first adjusted to 1 mM by ultrafiltration through YM10 filters (Amicon Corp., Lexington, MA). Samples were subsequently equilibrated with D₂O by four cycles of buffer exchange using Centri-prep 10 concentrators (Amicon Corp.) The final buffer composition for NMR studies was 10 mM potassium phosphate, 5 mM 2-mercaptoethanol, and 0.05% azide. CRBP II was identified in column fractions by 15% polyacrylamide-sodium dodecyl sulfate gel electrophoresis (Li et al., 1987).
the absence of proton decoupling. Ninety degree flip angle pulses (6.8 μs) were employed. The 3P chemical shift was referred to a standard, the potassium salt of trifluoroacetic acid, which was included in each sample at a concentration of 1 mM. The 3P spin-lattice relaxation time (T1) was estimated by the inversion recovery method. The T1 for protein resonances was 0.4 s and for trifluoroacetic acid, 2 s. Spectra used for intensity measurements comparing protein and trifluoroacetic acid resonances were obtained with long delays (9 s) between pulses. However, most studies employed 1.2 s delay times between transients (~3 T1 values for protein resonances).

Molecular Model Building of CRBP II—Modeling studies were initiated using the refined atomic coordinates of crystalline E. coli-derived rat 1-FABP (Sacchettini et al., 1989) as a template and the primary structure alignment of Jones et al. (1988). These modeling experiments were carried out in four major steps using the computer graphics package SYBIL (Tripos Assoc.) on an Evans and Sutherland PS 390 graphics workstation. The first step was to replace the side chains of 1-FABP with those of CRBP II based on the aforementioned alignment. The second step was to energy refine the positions of the newly placed atoms while restraining any movement of the side chains of nonreplaced residues and main chain atoms. This energy refinement and all successive energy refinement procedures utilized the Kollman force field to minimize the strain energy of the molecule and were run to convergence. The next step was to build insertions and remove deletions that occur between the 1-FABP and CRBP II. This was again followed by energy refinement but only on the areas of rebuilt insertions and deletions. One final cycle of energy refinement was carried out with no constraints applied to the model.

RESULTS AND DISCUSSION

Synthesis of 6-Fluorotryptophan-labeled CRBP II (6-F-Trp-CRBP II)—CRBP II was labeled with 6-fluorotryptophan using the method developed by Ho and co-workers (Robertson et al., 1977). By growing a tryptophan auxotroph of E. coli transformed with the prokaryotic expression vector pMON-CRBP II in the presence of fluorotryptophan analogs, we were able to recover large amounts of fluorotryptophan-labeled CRBP II (5-10 mg of purified protein/1 of culture). The degree of substitution of analog for L-tryptophan in the purified protein was estimated to be 93%. This value was based on a comparison of the NMR signal area (by integration) between known amounts of 6-fluorotryptophan-labeled CRBP II and a standard solution of trifluoroacetic acid which was included as an internal standard.

Fluorescence Properties of Apo- and Holo-6-F-Trp-CRBP II—To determine whether incorporation of 3F significantly affected ligand binding, we compared the retinol binding properties of apo-6-F-Trp-CRBP II and unlabeled apo-CRBP II using fluorescence spectroscopy. As shown in Fig. 1A, addition of increasing amounts of all-trans-retinol to both labeled and unlabeled apo-CRBP II increases retinol fluorescence since the ligand binding sites are saturated. Conversely, protein fluorescence decreases until the ligand binding sites are saturated (Fig. 1B). The Kd values of the labeled and unlabeled proteins for all-trans-retinol were 5 ± 1 × 10^-8 M and 1.5 ± 0.3 × 10^-7 M, respectively (at a CRBP II concentration of 2 μM). The fluorescence excitation spectrum of 6-F-Trp-CRBP II titrated to saturation with all-trans-retinol exhibits fine vibronic structure between 300 and 390 nm which is similar to that observed for unlabeled CRBP II complexed with all-trans-retinol (Fig. 2). This observation suggests that the conformation of retinol bound to both proteins is more planar than the average conformation of retinol in solutions of organic solvent (Chytil and Ong, 1984). A broad peak in the fluorescence excitation spectrum occurs between 280 and 290 nm for both holo-CRBP II and holo-6-F-Trp-CRBP II. This peak probably represents energy transfer from tryptophan residue(s) to protein-bound all-trans-retinol (Chytil and Ong, 1984). The peak is smooth in the holo-6-F-TRP spectra as opposed to the peak in spectrum from unlabeled holo-CRBP II which has a shoulder at 290 nm (see arrow in Fig. 2). A comparison of the UV absorption spectra of 6-fluorotryptophan and tryptophan reveals analogous differences in their spectra between 280 and 290 nm (data not shown).

Together these observations suggest that the small, yet reproducible, differences in the fluorescence excitation spectra of holo-6-F-Trp-CRBP II and holo-CRBP II could "simply" reflect differences in the intrinsic UV absorption properties of tryptophan and the 6-fluorotryptophan analog. In summary, it appears that incorporation of the 3F spin label does not significantly affect the retinol binding properties of CRBP II.

3F NMR Spectra of Apo-6-F-Trp-CRBP II and 6-F-Trp-CRBP II Complexed with All-trans-retinol—The top panel of Fig. 3 shows the 3H coupled 470.3 MHz 3F NMR spectrum of a 1 mM solution of apo-6-F-Trp-CRBP II at 22 °C. Six peaks are present in the form of three doublets. Their chemical shifts relative to trifluoroacetic acid were -45.04 and -45.21 ppm (doublet AB in Fig. 3), -46.23 and -46.63 ppm (doublet C), and -47.70 and -47.83 ppm (doublet D). The AB, C, and D doublets have an integrated area ratio of 2:1:1. The line shape of the resonances was not affected by varying the protein concentration from 0.1 to 1.0 mM, suggesting that there is no appreciable self-aggregation of CRBP II at the concentrations used for these NMR studies.

The 3F NMR spectrum was dramatically modified by adding increasing amounts of all-trans-retinol (middle and lower panels of Fig. 3). The signals at -45.04 and -45.21 ppm (AB) were replaced by a broad singlet (A) at -43.28 ppm and a broad doublet (B) at -44.40 and -44.57 ppm. In contrast, the signals in doublet C and doublet D underwent only minor


**Fig. 7. Molecular model building of CRBP II.** Construction of CRBP II was performed as described under "Experimental Procedures" using the refined atomic coordinates of rat I-FABP as a template (Sacchettini et al., 1989) plus the primary structure alignment of rat CRBP II, rat I-FABP, and the P2 protein from bovine peripheral nerve (Jones et al., 1988). Panel A, alignment of the primary structures of CRBP II, I-FABP, and P2; panel B, stereodiagram of the predicted C, structure of rat CRBP II. The predicted positions of Met', Trp', Trp'', and Trp''' in rat CRBP II are also shown.

Perturbations in chemical shifts: doublet C shifted less than 0.2 ppm downfield to -46.10 and -46.47 ppm, whereas doublet D shifted less than 0.1 ppm upfield to -47.77 and -47.87 ppm. Under conditions of full saturation, singlet A and the three doublets B, C, D have an integrated area ratio of approximately 1:1:1:1. Examination of the spectrum obtained when only half of the CRBP II protein was complexed with all-trans-retinol confirms that there is a change in the chemical shift of these signals, since separate resonances were detected for the holo- and apoproteins (see middle panel of Fig. 3). The detection of separate resonances for holo- and apoproteins when half of the CRBP II protein was complexed with all-trans-retinol also suggests that the on/off rate of the ligand is slow on the NMR time scale. The upper limit of the exchange between holo- and apoprotein was estimated to be 38 s⁻¹ based on differences between the chemical shifts of the two resonances highlighted by arrows in the middle panel of Fig. 3.

Although the ¹⁹F NMR spectrum of holo-6-F-Trp-CRBP II at 22 °C indicated that resonances corresponding to W₄, W₅, and W₆, each consist of two components of unequal intensity (see Fig. 3, bottom panel), W₄ was represented by a single peak. However, when the spectrum was collected at 4 °C, resonance A becomes a doublet (see arrows in lower right panel of Fig. 4). These two components appear to coalesce at 10 °C. Although the resonances in each of the other three doublets (B,C,D) move closer together with increasing temperature, coalescence was not observed between 4 and 60 °C. The line widths of all of the peaks decreased with increasing...
temperature, consistent with a decrease in molecular correlation times. In summary, comparison of the $^{19}$F NMR spectra of holo- and apo-CRBP II suggested that the physical environment of 2 of the 4 tryptophan residues represented by resonances A and B ($W_A$ and $W_B$) were altered by binding all-trans-retinol. In contrast, the physical environment of the other 2 tryptophan residues represented by resonances C and D ($W_C$ and $W_D$) appeared to be relatively unaffected by ligand binding.

**Evidence That the Initiator Methionine Residue Affects the Local Environment of All 4 Tryptophan Residues**—The finding of more than one resonance per tryptophan residue was not unique of the 6-fluorotryptophan analog preparation used. It was also observed for 4-fluorotryptophan-labeled CRBP II and 5-fluorotryptophan-labeled CRBP II, although the $^{19}$F NMR spectra were not as well resolved as with 6-fluorotryptophan-substituted protein (data not shown).

Detection of resonances in the form of "doublets" corresponding to a single tryptophan residue has been reported previously by Kimber et al. (1977) in the $^{19}$F NMR spectrum of 6-F-Trp dihydrofolate reductase from *Lactobacillus casei*, and also by Groff et al. (1981) in the $^{13}$C NMR spectrum of the same enzyme labeled with $[\gamma,13C]$tryptophan.

The two major explanations for detection of more than one resonance corresponding to a single tryptophan residue in 6-F-Trp-CRBP II are (i) chemical exchange of the tryptophan residues between alternative microenvironments and (ii) chemical heterogeneity in the protein sample (Schaefer et al., 1989) where the microenvironments of the tryptophan residues differ between the protein species. To address possibility ii, we subjected two of our purified preparations of 6-F-Trp-CRBP II to automated sequential Edman degradation. Each preparation exhibited NH$_2$-terminal heterogeneity. Our initial prep, used for the studies described in Figs. 3 and 4, consisted of two species: 62% of the E. coli-derived 6-F-Trp-CRBP II had retained its NH$_2$-terminal initiator Met (Met$^+$ CRBP II), whereas 38% had an NH$_2$-terminal Thr (Met$^-$ CRBP II). This Thr represents residue 2 of the primary translation product. Met$^+$ CRBP II probably arises from co- or early post-translational processing by *E. coli* aminopeptidases (Brown, 1973). A subsequent preparation of *E. coli* derived 6-F-Trp-CRBP II had a different composition: 45% Met$^+$ and 55% Met$^-$ (see Footnote 3). In order to assess the effects this NH$_2$-terminal heterogeneity on the $^{19}$F NMR spectra, we compared the spectrum of this preparation of 6-F-Trp-CRBP II complexed with all-trans-retinol with the spectrum obtained from our initial preparation. The upper panel of Fig. 5 presents the $^{19}$F NMR spectrum obtained at 22 °C from the sample of 6-F-Trp-CRBP II that had a Met$^+/Met^-$ ratio of 45/55, whereas the bottom panel shows its 4 °C spectrum. The comparison revealed that there was an alteration in the relative intensities of the two components of the B, C, and D resonances which corresponded to the different ratios of Met$^+$ and Met$^-$ CRBP II species in the two preps. Thus, these results suggest that the detection of more than one resonance corresponding to a single tryptophan residue is due to chemical (NH$_2$-terminal) heterogeneity in the protein sample. Based on the $^{19}$F spectra obtained from the two preparations of *E. coli* 6-F-Trp-CRBP-II, we assigned the downfield component of the B resonances at -44.42 ppm, the upfield component of the C resonances at -46.47 ppm, and the downfield component of the D resonances at -47.78 ppm to the Met$^+$ species (see Fig. 5). Although the results of the temperature study on the initial Met$^+$-enriched CRBP II preparation (Fig. 4) appear consistent with the model that $W_A$, the tryptophan corresponding to resonance A, is in intermediate to fast exchange between two microenvironments at temperatures greater than 10 °C, comparison of its 4 °C spectrum with the 4 °C spectrum of the Met$^-$-enriched sample (lower panel of Fig. 5) indicates that this is not the case. In the sample with less Met$^+$-CRBP II, the downfield component of resonance A located at -42.96 ppm is no longer seen as a discrete peak but rather as a shoulder in a broad peak at -43.23 ppm (lower panel of Fig. 5). Based on this comparison of the two preps, we

<table>
<thead>
<tr>
<th>Tryptophan residue</th>
<th>Environment</th>
<th>Predicted resonances</th>
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<tbody>
<tr>
<td>9</td>
<td>Partly buried in hydrophobic region</td>
<td>B</td>
</tr>
<tr>
<td>89</td>
<td>Relatively more exposed to solvent</td>
<td>C</td>
</tr>
<tr>
<td>107</td>
<td>Buried in hydrophobic region in substrate pocket</td>
<td>A</td>
</tr>
<tr>
<td>110</td>
<td>Relatively more exposed to solvent and adjacent to very polar groups (Arg$^{99}$, Thr$^{102}$, Lys$^{106}$, Glu$^{111}$, Gly$^{113}$)</td>
<td>D</td>
</tr>
</tbody>
</table>

$^{3}$ Attempts to further convert Met$^+$ CRBP II to Met$^-$ CRBP II by digestion with both cytosolic leucine aminopeptidase (EC 3.4.11.1) and microsomal leucine aminopeptidases (EC 3.4.11.2) were unsuccessful. Repeated efforts to purify Met$^+$ CRBP II from Met$^-$ CRBP II using methods that were utilized previously to separate the homologous Met$^+$ and Met$^-$ *E. coli*-derived CRBP species (Levin et al., 1989) were also unsuccessful.
concluded that the apparent doublet in resonance A, revealed in the 4 °C spectrum of our initial prep of 6-F-Trp-CRBP II, also arises from NH2-terminal heterogeneity and have assigned the downfield component to the Met* species and the upfield component to the Met species.

In summary, the presence or absence of the initiator Met residue affects the NMR spectral parameters of the fluorine nuclei on all 4 tryptophan residues. It has the largest effect on Wc, since the two components of its resonance have the largest difference in chemical shifts (0.58 ppm). It has the least effect on Wa, since the A resonance appears as a singlet at room temperature and separates into two components only when the temperature is lowered to 4 °C.

19F NMR Spectrum of 6-F-Trp-CRBP II Complexed with All-trans-retinal—Previous fluorescence experiments have shown that intestinal and E. coli-derived apo-CRBP II both form high affinity complexes with all-trans-retinal (MacDonald and Ong, 1987; Levin et al., 1988). The lack of vibronic fine structures in the CRBP II-retinal spectrum, as opposed to the CRBP II-retinol spectrum, led MacDonald and Ong (1987) to propose that all-trans-retinol does not interact with the binding site of CRBP II in the same manner of all-trans-retinol. Fig. 6 shows a 470.3 MHz 19F NMR spectrum (collected at 22 °C) of a 0.26 mM solution of 6-F-Trp-CRBP II complexed with all-trans-retinol. Seven major peaks are present in the form of one singlet and three doublets with chemical shifts relative to trifluoroacetic acid of -42.46 (A), -44.05, and -44.18 ppm (B), -46.27 and -46.67 ppm (C), and -47.74 and -47.85 ppm (D). This spectrum is similar in appearance to the spectrum of 6-F-Trp-CRBP II complexed with all-trans-retinol (see Fig. 3, bottom panel). Thus, binding of all-trans-retinol results in perturbation of the same resonances as does 6-F-Trp-CRBP II binding of all-trans-retinol. Moreover, the direction of the changes in chemical shifts of resonances A and B are the same (both downfield). The major difference is that binding of all-trans-retinal results in a further downfield shift in the resonance corresponding to WA (2.6 ppm) than binding of retinol (2.0 ppm). The similarity of the NMR spectra of 6-F-Trp-CRBP II retinal and 6-F-Trp-CRBP II retinol suggest that the physical environments of the 4 tryptophan residues are affected in a similar manner by these retinoids. Therefore, we predict that all-trans-retinol and all-trans-retinal are bound at the same site, in the same orientation and/or induce similar conformational changes in the protein. Moreover, as noted in the Introduction, qualitative differences noted previously (MacDonald and Ong, 1987) in the contours of the absorption spectra of CRBP II retinol and CRBP II retinal must be viewed with caution since these differences may be related to [Π]/[Π*]/n/Π* mixing rather than differences in protein-ligand interactions (Levin et al., 1988).

Correlation of 19F NMR Spectra of 6-F-Trp-CRBP II with a Three-dimensional Model of Its Structure—E. coli-derived apo-CRBP II has recently crystallized (Sacchettini et al., 1987). However, its tertiary structure has not yet been defined. Rat CRBP II belongs to a family of 10 small cytoplasmic proteins that bind hydrophobic ligands (Jones et al., 1988; Matareese and Bernholz, 1988; Waiz et al., 1988). Rat intestinal fatty acid binding protein (I-FABP) and the bovine myelin P2 protein represent the only two family members for which such information is available (Sacchettini et al., 1988; Jones et al., 1988). Both proteins consist of two, nearly orthogonal, β sheets formed by 10 anti-parallel β strands. The structure resembles a clam shell and has been named a β-clam (Sacchettini et al., 1989). Their bound long chain fatty acids are located in the interaction of the β-clam. Despite the fact that I-FABP and P2 share only 28% primary sequence identity, their Cα chains are virtually superimposable (r.m.s. difference < 1.8 Å). Therefore, it is reasonable to assume that CRBP II, which shares a similar degree of identity to these two other proteins (Fig. 7A), would share a similar tertiary structure (see Jones et al., 1988 for a discussion of this hypothesis). The structural alignment of CRBP II, I-FABP, and P2 proposed by Jones et al. (1988) predicted that Trp5, Trp8, Trp107, and Trp110 of CRBP II are in β-strands. We used the primary structural alignment proposed by Jones et al. (1988) plus the refined atomic coordinates of crystalline E. coli-derived rat I-FABP (Sacchettini et al., 1989) to initiate molecular modeling of the tertiary structure of rat CRBP II. The methods employed for model construction are described under “Experimental Procedures” and the results presented in Fig. 7B. The locations of the 4 Trp residues in our model of CRBP II are consistent with the predictions made by Jones and co-workers (1988). Trp5, which corresponds to Trp of the P2 protein and Trp0 of I-FABP (Fig. 7A), would be pointing into the β-clam but is still partially exposed to solvent (Fig. 7B). Trp8, which corresponds to Leu88 of P2 protein and Met46 of I-FABP, would point away from the β-clam and be relatively more exposed to solvent. Trp107, which corresponds to Ile119 of P2 protein and Ala169 of I-FABP are located in their ligand binding sites. Suggests that Trp107 may also be in the ligand binding site of CRBP II. Trp110 of CRBP II, which corresponds to Lys167 of P2 protein and Gin137 of I-FABP, is predicted to be pointing away from the β-clam and adjacent to a number of polar groups including Asn130, Thr118, Lys126, and Glu112 (see panels A and B of Fig. 7).

The theoretical basis for the shielding of the 19F nucleus in a protein is complex. Multiple effects may be involved, including electric fields arising from polar or charged groups, van der Waals forces, as well as ring currents from the side chains of neighboring amino acids and from the bound ligand. Based on empiric measurements, Robertson et al. (1977) suggested that a downfield shift indicates that the fluorine nucleus is in a more hydrophobic environment. Kimber et al. (1977) predicted that since the second-order electric field contribution leads to downfield shifts, resonances arising from “buried” fluoro-amino acids in proteins will appear downfield of the corresponding group in a more solvent-accessible environment.

Fig. 8 shows that after the denaturation of 6-F-Trp-CRBP II in 4 m guanidine HCI, all the 19F signals collapse into a sharp feature with chemical shifts of the primary signal at -46.37 ppm and a partially resolved shoulder at ~46.43 ppm. These values were obtained from the denatured protein, all of whose 6-fluorotryptophan residues are exposed to solvent, and are virtually identical to the chemical shift of free 6-fluorotryptophan (data not shown). The data from the denatured protein can be used as a reference point in assigning resonances to specific Trp residues in the native protein. Using the predicted three-dimensional structure of CRBP II plus the conclusions of Robertson et al. (1977) and Kimber et al. (1977) described in the previous paragraph, we propose the following testable hypothesis: that WA corresponds to Trp5, Wa to Trp8, Wc to Trp107, and Wo to Trp110. Since the corresponding resonances are downfield of the signal from denatured CRBP II and demonstrate the largest change in chemical shift on ligand binding. It is relatively unaffected by the initiator Met residue. Wa is assigned to Trp5, since its corresponding resonances are also downfield from that of denatured CRBP II but shift less dramatically than that of Wc. Wc is assigned to Trp8 because
the chemical shifts of its corresponding resonances are near that of denatured CRBP II. The Wc resonances also appear most affected by the initiator Met residue. This is consistent with the predicted proximity of Trp80 to Met1 (see Fig. 7B). Wc is assigned to Trp80, since it is predicted to be adjacent to a number of polar groups. This could explain the position of its corresponding resonances upfield from that of denatured CRBP II. These assignments, which are summarized in Table I, can be tested by constructing mutant CRBP II proteins in which tryptophan residues have been replaced by other hydrophobic amino acids.

In conclusion, the site-specific changes in chemical shifts of resonances, detected in the 1H NMR spectra of 6-F-Trp-CRBP II upon binding of retinol and retinal, indicated that 1H nuclei provide sensitive probes for CRBP II-ligand interactions. The data suggest that NMR analysis of isotopically labeled recombinant proteins expressed in E. coli may provide a useful method for investigating protein-ligand interactions in other members of this protein family.

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