It has been suggested that electrostatic interactions are critical for binding of retinoic acid by cellular retinoic acid-binding proteins (CRABP-I and CRABP-II). However, the roles of two conserved arginine residues (Arg-111 and Arg-131 in CRABP-I; Arg-111 and Arg-132 in CRABP-II) that interact with the carboxyl group of retinoic acid have not been evaluated. A novel competitive binding assay has been developed for measuring the relative dissociation constants of the site-directed mutants of CRABPs. Arg-111 and Arg-132 of CRABP-II were replaced with methionine by site-directed mutagenesis. The relative dissociation constants of R111M and R132M (K_d[R111M]/K_d[CRABP-II], and K_d[R132M]/K_d[CRABP-II]) were determined to be 40–45 and 6–8, respectively. The ring protons of the aromatic residues of the wild-type CRABP-II and the two mutants were sequentially assigned by two-dimensional homonuclear NMR in conjunction with three-dimensional heteronuclear NMR. Detailed analysis of the nuclear Overhauser effect spectroscopy spectra of the proteins indicated that the conformations of the two mutants are highly similar to that of the wild-type CRABP-II. These results taken together showed that Arg-111 and Arg-132 are important for binding retinoic acid but contribute to the binding energy only by ~2.2 and 1.2 kcal/mol, respectively. In addition, the relative dissociation constant of CRABP-II and CRABP-I (K_d[CRABP-II]/K_d[CRABP-I]) was determined to be 2–3, in close agreement with that calculated using the apparent K_d values determined under the same conditions by fluorometric titrations.

Retinoic acid (RA), a hormonally active metabolite of vitamin A, has profound effects on cell growth, differentiation, and morphogenesis. Two types of proteins have been found to bind RA: nuclear retinoic acid receptors (RARs and RXRs) and cellular retinoic acid-binding proteins (CRABPs). RARs and RXRs are RA-activated transcriptional factors that regulate expression of target genes (1). Although the physiological roles of CRABPs are not clear at present, they are thought to be involved in cellular transport and metabolism of RA (2). Two isoforms (CRABP-I and CRABP-II) have been characterized. Both CRABP-I and CRABP-II bind specifically to all-trans-retinoic acid, but they differ in several respects: (i) CRABP-I has higher affinity for RA than CRABP-II (3–6); (ii) CRABP-I is expressed in many adult mouse and human tissues, but the expression of CRABP-II is limited to skin (7, 8); and (iii) RA stimulates expression of CRABP-II but not that of CRABP-I (8). It appears that the two isoforms may have distinct functions. The idea is supported by the fact that the sequence identity of human and mouse CRABP-I (99.3%) or human and mouse CRABP-II (93.5%) is much higher than the sequence identity (73.7%) between the two isoforms from the same source.

CRABPs are members of a family of intracellular lipid-binding proteins that bind small hydrophobic molecules such as retinoids and fatty acids (9). The family of proteins includes CRABPs, cellular retinol-binding proteins, fatty acid-binding proteins, P2 myelin protein, a mammary gland protein, and gastrin. The structures of 11 different intracellular lipid-binding proteins, including CRABP-I and CRABP-II, have been determined by x-ray crystallography (9–11). Although the sequence identity among intracellular lipid-binding proteins is rather low (~20%), these crystal structures are remarkably similar with respect to backbone folding. They are composed of two nearly orthogonally packed five-stranded β-sheets and two short α-helices. The ligand binding pocket in each protein is deep inside the interior of the β-barrel formed by the two β-sheets. The helix-turn-helix motif is located at the ligand entrance. These proteins apparently lack a true hydrophobic core that is important for folding and stability of many other proteins (12).

Electrostatic interactions are thought to play major roles in binding of RA by CRABPs because the two proteins bind only retinoic acid but not retinol and retinal. Two arginine residues (Arg-111 and Arg-131 in CRABP-I; Arg-111 and Arg-132 in CRABP-II) have been identified by crystallography to interact with the carboxyl group of the bound RA (10). However, previous site-directed mutagenesis studies suggest that both Arg-111 and Arg-131 are critical for CRABP-I to bind RA (13) but that only Arg-132 is important for CRABP-II to bind RA (14). Because of the limitations of the current methods for measuring RA binding, the dissociation constants of the critical mutants have not been measured. Furthermore, the conformations of the CRABP-I mutants have been characterized only by CD, and the conformations of the CRABP-II mutants have not been characterized. Thus, the contributions of the electrostatic interactions between the arginine residues and RA to binding are still uncertain.

We are interested in studying the quantitative structure-function relationships of CRABPs with respect to binding of...
RA. In this paper, we report a novel competitive binding assay developed for measuring the dissociation constants of the site-directed mutants of CRABPs. We have used the novel method to evaluate the contributions of both Arg-111 and Arg-132 of CRABP-II to binding of RA in conjunction with site-directed mutagenesis and NMR. The results show that like CRABP-I, both Arg-111 and Arg-132 in CRABP-II are important for binding of RA, contrary to the results of Chen et al. (14). However, Arg-111 and Arg-132 contribute to the overall binding energy only by 2.2 and 1.2 kcal/mol, respectively.

EXPERIMENTAL PROCEDURES

Materials—Nonradioactive RA was purchased from Sigma. (11, 12-2H)RA was purchased from DuPont NEN. A DNA sequencing kit was obtained from U.S. Biochemical Corp. Enzymes for recombinant DNA experiments were purchased from Life Technologies, Inc., or New England Biolabs. Other chemicals were analytical or reagent grade from commercial sources.

Cloning and Expression—The cDNAs encoding human CRABP-I and CRABP-II were kindly provided by Dr. Anders Åstro¨m (8). The genes were subcloned into the expression vector pET-17b (Novagen) by polymerase chain reaction. The primers for polymerase chain reaction were: 5'-CCATGGATTGCTGGCAGGACAAGCAATG-3' (forward, CRABP-I), 5'-CGGGATCCTCATTCCGGGACATAAATTC-3' (reverse, CRABP-I), 5'-CGGATCCATATGGCAATCTGGCAGGACAAGCAATG-3' (forward, CRABP-II), and 5'-GGATATTCCTACTCTCGACGATGAC-3' (reverse, CRABP-II). To ensure that there were no undesired mutations in the amplified genes, they were sequenced by double-stranded DNA sequencing from both orientations. The expression constructs were then transformed into the bacterial strain BL21(DE3)pLysS (15). The transformed bacterial cells were grown at 37 °C in a LB agar plate containing both ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml). Expression of CRABPs was verified by growing the colonies in 5 ml of LB medium followed by induction with isopropyl-1-thio-β-D-galactopyranoside and SDS-polyacrylamide gel electrophoresis of the harvested cells.

Site-directed Mutagenesis—The oligonucleotides for making human CRABP-II mutants were 5'-CTGTTGGCACATGGCAGGACA-3' (R111M) and 5'-GTGTTGGACATGGCAGGACA-3' (R132M). The mutated plasmids were subcloned into the expression vector pET-17b (Novagen) by polymerase chain reaction. The primers for polymerase chain reaction were: 5'-CCATGGATTGCTGGCAGGACAAGCAATG-3' (forward, CRABP-I), 5'-CGGGATCCTCATTCCGGGACATAAATTC-3' (reverse, CRABP-I), 5'-CGGATCCATATGGCAATCTGGCAGGACAAGCAATG-3' (forward, CRABP-II), and 5'-GGATATTCCTACTCTCGACGATGAC-3' (reverse, CRABP-II). To ensure that there were no undesired mutations in the amplified genes, they were sequenced by double-stranded DNA sequencing from both orientations. The expression constructs were then transformed into the bacterial strain BL21(DE3)pLysS (15). The transformed bacterial cells were grown at 37 °C in a LB agar plate containing both ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml). Expression of CRABPs was verified by growing the colonies in 5 ml of LB medium followed by induction with isopropyl-1-thio-β-D-galactopyranoside and SDS-polyacrylamide gel electrophoresis of the harvested cells.

Protein Purification—All proteins were purified by the same procedure as described below. 100 ml of LB medium containing 100 μg/ml of ampicillin and 25 μg/ml of chloramphenicol was inoculated by a small piece of a frozen seed culture and incubated at 37 °C with vigorous shaking (200 rpm) overnight. It was then used to inoculate 4 liters of LB medium containing both antibiotics and grown at 37 °C. When A600 of the culture reached 0.6–0.8, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.4 mM. The culture was allowed to grow further at 30 °C for 3–5 h. The bacterial cells were then harvested by centrifugation and suspended in 100 ml of buffer A (10 mM Tris-HCl, 1 mM diithiothreitol, pH 8.2). The suspension was sonicated on ice and centrifuged (27,000 × g) at 4 °C for 30 min. The supernatant was applied to a DEAE-cellulose column equilibrated with the same buffer. The column was washed with buffer A until A600 of the eluent was less than 0.05. Elution of the column was achieved by a linear NaCl gradient (0–200 mM in buffer A) and monitored by absorbance at 280 nm. The fraction containing CRABP was pooled and concentrated by an Amicon ultrafiltration cell using a YM 10 membrane. The concentrated protein solution was centrifuged (27,000 × g) for 20 min. The supernatant was loaded onto a Sephadex G-50 column equilibrated with phosphate-buffered saline (4 mM NaH2PO4, 150 mM NaCl, pH 7.3) and eluted with the same buffer. The fractions containing CRABP were pooled by A280 and 15% SDS-polyacrylamide gel electrophoresis. The fractions containing greater than 99% purity were pooled and concentrated. The protein solution was dialyzed against double-distilled water and lyophilized.

Fluorometric Titration—Fluorescence binding assays were carried out by a procedure modified from that of Cogan et al. (17) using a Hitachi 4500 fluorometer. Brieﬂy, CRABPs were dissolved in phosphate-buffered saline. The concentrations of the protein stock solutions were measured by A280. The absorption coefﬁcients used were 19,688 m-1 cm-1 for CRABP-I and 19,480 m-1 cm-1 for CRABP-II determined by the method of Gill and Hippiel (18). RA stock solutions were prepared in absolute ethanol. The concentrations of the RA stock solutions were determined by A280 using the absorption coefficient of 45,000 m-1 cm-1. The ﬁnal ethanol content for each titration was kept less than 2%. The samples were excited at wavelength of 283 nm with a slit width of 2.5 nm. The emission wavelengths were 330 nm for CRABP-I and 338 nm for CRABP-II with a slit width of 20 nm. The excitation shutter was closed between measurements. The inner filter corrections were not corrected because they were negligible at the protein and RA concentrations we used in the experiments. The data were analyzed by nonlinear least square ﬁt to Equation 1.

\[
F/F_0 = 1 + \left( \frac{P_1 + K_d}{K_d} \right) \times \left( \frac{P_1 + K_d}{2P_1} \right) \tag{1}
\]

where F is the observed ﬂuorescence, F0 is the ﬂuorescence of the bound CRABP, P1 is the total protein concentration, and R1 is the total RA concentration.

Competitive Binding Assay—The assay was carried out in a Spectra/Par equilibrium dialyzer at room temperature. The two compartments of each dialysis cell were separated by a semipermeable membrane with a molecular mass cut-off of 6–8 kDa. One compartment was ﬁlled with the wild-type CRABP-II (1 ml), and the other was ﬁlled with a CRABP-II mutant or the wild-type CRABP-I. The buffer used was phosphate-buffered saline plus 5 mM dithiothreitol. An equal amount of [3H]RA (100 nM) was added to each compartment. The protein concentrations in both compartments were much greater (at least 20-fold) than the RA concentration. 100 μl of samples was taken from the two compartments after various times of incubation at room temperature, mixed with 5 ml of scintillation ﬂuids, and counted by a liquid scintillation counter. The equilibria in the two compartments that contained the wild-type CRABP-II and mutant proteins can be described by Equations 2 and 3 (similar equations can be written for CRABP-I and CRABP-II).

\[
K_d (WT) = \frac{[RAT]}{[RA] \cdot [WT]} \tag{2}
\]

\[
K_d (MT) = \frac{[RAT]}{[RA] \cdot [MT]} \tag{3}
\]

Since the concentrations of the proteins were much greater than their respective dissociation constants and the concentration of RA, [WT] = [MT]total, [MT] = [WT]total >> [RA], and [MT]RA >> [RA], the relative dissociation constant can be calculated by Equation 5.

\[
K_d (MT) = \frac{[MT]_{total} \cdot [WT]}{[MT]_{total} \cdot [WT]} \tag{5}
\]

where CWT and CMT are the measured radioactivities of the two compartments containing the wild-type CRABP-II and the mutant, respectively. It turned out that the system could not reach equilibrium in 2 days, presumably because of few free RAs in solution to diffuse across the membrane. Since RA is not stable even in the dark, the assay was redesigned to match the equilibrium conditions by varying the ratio of the protein concentrations of the wild-type and the mutant (MTtotal/WTtotal). The concentration of the mutant was varied while keeping the concentration of the wild type at ~2 μM. Initially, the concentration of the mutant was increased in an exponential manner (e.g. 2, 20, 200 μM). Then it was varied in a small range. Since an equal amount of RA was added to the two compartments of the dialysis cell, the two compartments should have the same RA concentration and radioactivity at the beginning of the assay. If [MT]total/[WT]total ≠ K_d(WT)/K_d(MT), there would have a net transfer of RA across the semipermeable membrane separating the two compartments. Thus, the radioactivity counts of the two compartments (CWT and CMT) would differ after incubation for a certain period. When [MT]total/[WT]total < K_d(MT)/K_d(WT), then CWT > CMT > 0. When [MT]total/[WT]total > K_d(MT)/K_d(WT), then CWT < CMT < 0. When [MT]total/[WT]total = K_d(MT)/K_d(WT), then CWT = CMT = 0.

NMR Spectroscopy—All NMR measurements were performed at 32 °C on a Varian VXR-500 spectrometer operating at a proton frequency of 500 MHz. The proteins were dissolved in 20 mM sodium phosphate, pH 7.5 (direct pH meter reading), 100 mM NaCl, 5 mM diethiothreitol in D2O. The protein concentration was 2–5 μM. The spec
results. For wild-type CRABP-II, one phase-sensitive DQF-COSY spectrum (19), two MLEV-17 clean TOCSY spectra (20–22) with mixing times of 20 and 40 ms and one NOESY spectrum (23, 24) with a mixing time of 150 ms were acquired. For R111M, one DQF-COSY spectrum, three TOCSY spectra with mixing times of 20, 40, and 75 ms and one NOESY spectrum with a mixing time of 150 ms were acquired. For R132M, one TOCSY spectrum with a mixing time of 40 ms and one NOESY spectrum with a mixing time of 150 ms were acquired. All of the spectra were acquired in the hypercomplex mode with standard phase cycling schemes. The data were usually acquired with 2048 complex points in the r2 dimension and 256 complex points in the t1 dimension. 96 transients were collected for each FID. Data processing was performed on a Sun Sparc 10 station using VNMR software from Varian. The time domain data were zero-filled once and multiplied by shifted sine bell or Gaussian functions before Fourier transformation in both dimensions. Base lines were corrected in t2 dimensions using a 5-order polynomial. Chemical shifts were referenced to internal sodium 3-(trimethyl silyl)-propionate-2,2,3,3-\textit{d}_4.

**RESULTS**

Fluorometric Titration—Probably because of the simplicity of the method, fluorometric titration has been frequently used for measuring the affinities of CRABPs for RA. However, there are large discrepancies in the $K_d$ values obtained by the method. These could be due to variations in the assay methodology and/or conditions. Some problems with the method have been recently discussed (2, 4, 6). In order to compare the ligand binding properties of CRABPs under the same conditions, we carried out fluorometric titration experiments. The results are shown in Fig. 1. The apparent $K_d$ values obtained by nonlinear fitting were 0.63 nM for CRABP-I and 1.9 nM for CRABP-II. While our work was in progress, Norris et al. (6) reported the apparent $K_d$ values of mouse CRABPs measured by an improved fluorescence titration method. The major changes were the lowering of protein and RA concentrations and the use of nonlinear least square fitting for data analysis. These modifications were in line with our approach. Our fluorometric titration data were analyzed by nonlinear least square fitting. The protein concentrations we used in the assays (~50 nM) were the lowest among those used in previous studies except those of Norris et al. (6). We did not include the additive gelatin used by Norris et al. (6) in the assays. The apparent $K_d$ values determined by us are in close agreement with those (0.4 nM for CRABP-I and 2 nM for CRABP-II) reported by Norris et al. (6).

Competitive Binding Assay—In order to measure the affinities of the mutants of CRABPs for RA, we developed a novel competitive binding assay. We first used the method to measure the relative affinity of CRABP-I and CRABP-II for RA. The result is shown in Fig. 2A. When the ratio of the concentrations of the two proteins ([CRABP-II]/[CRABP-I]) was =2, there was a net transfer of RA from the compartment containing CRABP-II to the compartment containing CRABP-I. When the ratio of the concentrations of the two proteins was =3, there was a net transfer of RA from the compartment containing CRABP-I to the compartment containing CRABP-II. As described under “Experimental Procedures,” the relative $K_d$ of the two proteins lies between the points with opposite net transfers. Thus the $K_d$ of CRABP-II relative to that of CRABP-I ($K_d$(CRABP-II)/$K_d$(CRABP-I)) is 3–2. It is in close agreement with the results of the fluorometric titration studies. We then used the competitive binding assay to measure the $K_d$ values of R111M and R132M relative to that of the wild-type CRABP-II. The results are shown in Fig. 2B and C. In the case of R111M (Fig. 2B), there was a net transfer of RA from the compartment containing R111M to the compartment containing the wild-type CRABP-II when [R111M]/[WT] ≥ 40. When [R111M]/[WT] ≥ 45, there was a net transfer of RA from the compartment containing the wild-type protein to the compartment containing R111M. Therefore, the relative affinity of R111M for RA

![Fig. 1. Fluorometric titration of wild-type CRABP-I (A) and CRABP-II (B). The concentrations of CRABP-I and CRABP-II were 40 and 50 nM, respectively.](image)

(K$_d$(R111M)/K$_d$(WT)) is 40–45. As shown in Fig. 2C, the relative affinity of R132M for RA (K$_d$(R132M)/K$_d$(WT)) is 6–8.

Sequential Resonance Assignment of the Ring Protons of the Aromatic Residues of the Wild-type CRABP-II and Site-directed Mutants—Since perturbations in the ligand binding property of a mutant may be due to conformational changes caused by the mutation (25), we characterized the conformations of the mutants by NMR. A prerequisite for NMR structural analysis is sequential resonance assignment. Currently, we are in the process of making total sequential resonance assignments of the wild-type CRABP-II and R111M by multidimensional multinuclear NMR. We have sequentially assigned the ring protons of aromatic residues of the wild-type and mutants based on the homonuclear two-dimensional NMR (DQF-COSY, TOCSY, and NOESY) and heteronuclear three-dimensional NMR. The chemical shifts of the ring protons are listed in Table I. The sequential resonance assignment is labeled in Fig. 3 for R132M. In comparison with the chemical shifts of the wild-type ring protons, there are quite a few chemical shift changes in the ring protons of the mutants. Some chemical shift changes can be easily rationalized, while the causes for other chemical shift changes are not obvious. For example, the guanidino group of Arg-132 stacks on the aromatic ring of

L. Wan, Y. Li, and H. Yan, unpublished results.
Phe-15 and is hydrogen-bonded to the hydroxyl group of Tyr-134 in the crystal structure of the wild-type CRABP-II (10). Removal of the guanidino group is likely to be the cause for the chemical shift changes in Phe-15 and Tyr-134 of R132M. The crystal structure also reveals that the guanidino group of Arg-132 contributes to the binding energy by 2.2 kcal/mol, respectively. The aromatic-aromatic NOEs shown in the upper panels of the figures are not known at present. However, qualitatively, the three proteins have very similar aromatic-aliphatic NOE patterns. The results of the NOESY analysis suggest that the conformations of R111M and R132M are highly similar to that of the wild-type protein. Thus, the decreases in affinity for RA of the two mutants are most likely due to the disruption of the interactions of the arginine residues with RA by mutation. Arg-111 and Arg-132 contribute to the binding energy by 2.2 and 1.2 kcal/mol, respectively.

**TABLE I**

| Residue | Phe-3 | Phe-15 | Phe-50 | Phe-65 | Phe-71 | Tyr-51 | Tyr-104 | Trp-7 | Trp-87 | Trp-87 | Trp-109 |
|---------|-------|--------|--------|--------|--------|--------|---------|--------|--------|--------|---------|
| Wild-type CRABP-II | 7.60 | 7.42 | 6.66 | 6.42 | 7.48 | 7.12 | 6.98 | 7.25 | 8.16 | 7.00 | 6.09 |
| R111M | 7.34 | 7.02 | 7.26 | 6.70 | 7.00 | 6.74 | 6.65 | 7.00 | 7.44 | 6.99 | 6.73 |
| R132M | 7.52 | 7.11 | 6.99 | 6.48 | 6.70 | 6.74 | 6.66 | 7.00 | 6.99 | 6.73 | 6.75 |
| a,b | 2,6H, 3,5H, and 4H of phenylalanine, respectively. | d,e | 2,6H and 3,5H of tyrosine, respectively. | f | 4H, 5H, 6H, and 7H of tryptophan, respectively. | g,h | Trp-87 has two sets of resonances because of conformational heterogeneity.

**FIG. 2.** Competitive binding assays for measuring the relative dissociation constants of CRABPs. The relative radioactivity in A is the radioactivity count of the compartment containing CRABP-I minus that of the compartment containing CRABP-II. The relative radioactivity in B is the radioactivity count of the compartment containing CRABP-II minus that of the compartment containing R111M. The relative radioactivity in C is the radioactivity count of the compartment containing CRABP-II minus that of the compartment containing R132M.

**Chemical shifts of the ring protons of the aromatic residues of wild-type CRABP II, R111M mutant, and R132M mutant**

The underlined values are the mutant resonances that differ by >0.02 ppm from the corresponding resonances of the wild-type CRABP-II.

**Competitive Binding Assay**—Two types of methods have been in general use for measuring binding of RA to CRABPs: fluorometry and radiometry. The radiometric method involves...
separation of bound from free RA by dextran-coated charcoal, gel filtration, and other means. Substantial loss of bound ligand during the separation process makes the method unsuitable for measuring the dissociation constants of site-directed mutants with greatly decreased affinity for RA. The very limited solubility of RA in water (−200 mM) (26) also makes the fluorometric method inapplicable for determining the dissociation constants of these mutants. Studies of the quantitative structure-function relationships of CRABPs have been hampered by the lack of methods for measuring the affinities of site-directed mutants for RA (13, 14). We have developed a novel competitive binding assay for measuring the affinities of site-directed mutants for RA relative to that of the wild-type CRABP. The essence of the method is to monitor the competition between a mutant and the wild-type protein for binding of limited RA. The method uses an equilibrium dialyzer. The two compartments of each dialysis cell are filled with the wild-type and mutant proteins, respectively. The absolute concentration of RA is not important as long as the concentration of free RA is much smaller than that of bound RA. There is no need to separate bound from free RA by dextran-coated charcoal, gel filtration, and other means. The transfer of RA from one compartment to the other is determined by measuring the radioactivities of the samples taken from the two compartments. The direction of the net transfer is dependent on the relative affinity of the two proteins. A wide range of apparent dissociation constants of the two proteins can be varied by more than 1,000-fold, and a relative dissociation constant of a point mutant is sufficient for estimating the energetic contribution of the amino acid residue to ligand binding (ΔΔG = RTln(K\text{d,mut}/K\text{d,wild})).

In addition to vitamin A, many other hydrophobic molecules such as vitamin D and steroid hormones play vital roles in a variety of cellular processes. Studies of the structure-function relationships of the receptor proteins for these bioactive hydrophobic molecules are of fundamental interest and biomedical significance. The method can also be used for measuring the relative dissociation constants of the site-directed mutants of these important proteins and may facilitate these studies.

Relative Affinity of CRABP-I and CRABP-II for RA—CRABP-I and CRABP-II differ in affinity for RA, expression pattern, and regulation. Although it is generally accepted that CRABP-I has a higher affinity for RA than CRABP-II, it has been difficult to determine precisely the relative affinity of the two proteins. A wide range of apparent K\text{d} values have been reported. The apparent K\text{d} values for purified CRABP-I are in the range of 0.4–39 nM (4–6, 13, 27–29). The apparent K\text{d} values for purified CRABP-II are in the range of 2–64 nM (9–14). The best estimation of the relative affinity of the two proteins for RA was made by Fiorella et al. (5). In their study, RA was first incubated with CRABP-II and then mixed with CRABP-I. The protein-RA complexes (CRABP-I-RA and CRABP-II-RA) were separated by chromatofocusing. The result indicated that CRABP-II has about 3-fold lower affinity for RA than CRABP-I. More recently, Norris et al. (6) compared the affinities of CRABP-I and CRABP-II for RA by fluorometric titration under the same conditions. The results showed that...
CRABP-I has 4-fold higher affinity for RA than CRABP-II (although a 10-fold stronger affinity of CRABP-I for RA, compared with CRABP-II, was stated in the paper, apparently due to an error in calculation). The apparent dissociation constant of CRABP-II relative to that of CRABP-I ($K_{d,CRABP-II}/K_{d,CRABP-I}$) is 3, based on our fluorometric measurements. The results of our competitive binding assays indicated that the relative apparent dissociation constant of CRABP-II ($K_{d,CRABP-II}/K_{d,CRABP-I}$) is 2–3. The results of our fluorometric measurements and competitive binding studies are in close agreement. They are also in line with the results of Fiorella et al. (5). The relative affinity of CRABP-I and CRABP-II measured by Fiorella et al. (5) may be the upper limit, because some RA bound to CRABP-II could be preferentially lost during separation of the protein-RA complexes by chromatofocusing. The difference in affinity for RA between CRABP-I and CRABP-II is rather small (1-2-fold).

Both Arg-111 and Arg-132 of CRABP-II Are Important for Binding of RA—Arg-111, Arg-132, and Tyr-134 of CRABP-II have been predicted to be involved in binding of RA on the basis of homologous modeling (13, 30, 31). The crystal structure of CRABP-II in complex with RA reveals that one of the carboxylate oxygens of RA is hydrogen-bonded to the guanidino group Arg-132 and the hydroxyl group of Tyr-134, and the other carboxylate oxygen of RA interacts with Arg-111 via a water molecule (10). We have substituted Arg-111 and Arg-132 with methionine. Our biochemical and structural characterizations show that guanidino groups of Arg-111 and Arg-132 contribute to the binding energy by −2.2 and 1.2 kcal/mol, respectively. The results indicate that both Arg-111 and Arg-132 are indeed involved in binding of RA and that Arg-111 contributes more to the binding energy than Arg-132. Fluorometric titration also suggests that R132M has higher affinity for RA than R111M (data not shown). Zhang et al. (13) have replaced the corresponding residues in CRABP-I with glutamine (R111Q and R131Q). Quantitative comparison of our results with those of Zhang et al. (13) is not possible, because the dissociation constants of their mutants have not been determined. Qualitatively, the ligand binding properties of our mutants are similar to those of the corresponding CRABP-I mutants. Zhang et al. (13) observed that both the wild-type CRABP-I and R131Q can induce CD of RA in the wavelength range between 320 and 360 nm, but R111Q cannot induce the CD signal. The results of the CD experiments with CRABP-I are consistent with our measurements of the energetic contributions of Arg-111 and Arg-132 to the binding of RA. Chen et al. (14) have mutated Arg-111 of CRABP-II to alanine (R111A) and Arg-132 of CRABP-II to alanine and glutamine (R132A and R132Q). In contrast to our R111M mutant, the apparent dissociation constant of their R111A mutant is very similar to that of the wild-type CRABP-II (only 50% higher than that of the wild type). The causes of the discrepancy are not clear, but several possibilities can be ruled out: (i) It is unlikely that there are mutations other than substitution of Arg-111 with a methionine in our R111M mutant. We have sequenced the entire gene after mutagenesis and subcloning into the expression vector. (ii) Steric hindrance is unlikely to be the cause, because the side chain of methionine is smaller than that of arginine. (iii) Although small conformational changes cannot be ruled out, NMR characterizations suggest that R111M is properly folded, and its conformation is
very similar to that of the wild-type protein. Furthermore, R111M has been crystallized. The preliminary crystal structure at 2.2-Å resolution shows that the structure of R111M is indeed very similar to that of the wild-type protein. The ligand binding property of our R132M mutant is qualitatively similar to that observed in the crystals. It is noted that the RA binding cavity is much larger than necessary to accommodate the ligand molecule (10). Our results agree with the crystallographic observation that both Arg-111 and Arg-132 are involved in binding of RA. However, in contrast to the crystallographic data, our mutagenesis results indicate that Arg-111 is more important than Arg-132 for RA binding. But this is not necessarily inconsistent with the crystallographic studies. Since Arg-132 and Tyr-134 are hydrogen-bonded to the same carboxylate oxygen of RA, the loss of the hydrogen bond between Arg-132 and RA in R132M may be partially compensated by the interaction between Tyr-134 and RA. On the other hand, the other carboxylate oxygen of RA only interacts with Arg-111 (albeit via a water molecule), the loss of the interaction in R111M may not be compensated. Alternatively, in solution, the position of the carboxyl group of the bound RA may be slightly different from that observed in the crystals. It is noted that the RA binding cavity is much larger than necessary to accommodate the ligand, and a large portion of the deep binding cavity is not occupied by RA. It appears that the β-ionone ring of the bound RA is well fixed, but the isoprene tail and carboxyl group have room to move. The guanidino group of Arg-111 could be closer to the carboxyl group of RA than that of Arg-132 in solution.

Solution NMR study of holo-CRABP-II in progress

CRABPs have very stringent retinoid specificity (2). The proteins bind only RA and reject both retinol and retinal. On the other hand, cellular retinol-binding proteins bind both retinol and retinal and reject RA. Amino acid sequence analysis reveals that the two conserved arginine residues in CRABPs are replaced with glutamine in cellular retinol-binding proteins. It has long been thought that the electrostatic interactions between the guanidino groups of the two conserved arginine residues and the carboxyl group of RA play major roles in binding of RA (31). Our mutagenesis studies indicate that Arg-111 and Arg-132 are indeed involved in RA binding, but the electrostatic interactions between the guanidino groups of Arg-111 and Arg-132 and the carboxyl group of RA contribute to the overall binding energy only by ~2.2 and 1.2 kcal/mol. Thus, amino acid residues that interact with the hydrophobic moiety of RA may be more important for binding of RA.

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