Fluorescence Studies of Rat Cellular Retinol Binding Protein II Produced in *Escherichia coli*: An Analysis of Four Tryptophan Substitution Mutants†

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ABSTRACT: Rat intestinal cellular retinol binding protein II (CRBP II) is an abundant 134-residue protein that binds *all-trans*-retinol which contains 4 tryptophans in positions 9, 89, 107, and 110. Our ability to express CRBP II in *Escherichia coli* and to construct individual tryptophan substitution mutants by site-directed mutagenesis has provided a useful model system for studying the fluorescence of a multi-tryptophan protein. Each of the four mutant proteins binds *all-trans*-retinol with high affinity, although their affinities are less than that of the wild-type protein. Steady-state and time-resolved fluorescence analyses of these proteins indicate that W107 is at the hydrophobic binding site, W110 is in a polar environment, and the remaining two tryptophans are in a hydrophobic environment. Time-resolved fluorescence study indicates that excited-state energy transfer occurs from the hydrophobic tryptophans to W110. The Stern–Volmer analysis with acrylamide of these proteins reveals that static quenching occurs in the W9F mutant protein while others do not. The fluorescence of rat intestinal fatty acid binding protein (I-FABP), a related protein of known X-ray structure, was also studied for comparison. The results of these findings, coupled with those derived from NMR studies and molecular graphics, suggest that CRBP II undergoes minor structural changes in all of the mutant proteins. Since these effects may be cumulative on the protein structure and function, any conclusions derived from higher mutants in this family of proteins must be treated with caution.

Rat cellular retinol binding protein II (CRBP II)[1] is an abundant 134-residue, intestinal protein that binds *all-trans*-retinol (Ong, 1984; MacDonald & Ong, 1987; Li et al., 1987). It belongs to the family of small cytoplasmic proteins which bind hydrophobic ligands (Matarese & Bernlohr, 1988; Sacchettini et al., 1990). Its primary structure has been determined from the nucleotide sequence of a cloned full length cDNA, and it contains four tryptophans at positions 9, 89, 107, and 110 (Li et al., 1986, 1987). Large amounts of purified apo-CRBP II free of ligand may thus be easily obtained. The *Escherichia coli* derived protein has ligand binding specificities and affinities that are indistinguishable from rat CRBP II isolated from rat intestine, implying that the recombinant protein is properly folded (Li et al., 1987).

Although CRBP II has been crystallized (Sacchettini et al., 1987), its molecular structure has not yet been determined. However, X-ray structures of two related members of this family of proteins, rat intestinal fatty acid binding protein (I-FABP) (Sacchettini et al., 1990) and P2 myelin (Jones et al., 1988), have been refined to 2.5 and 2.0 Å, respectively. Both proteins consist of two nearly orthogonal β-sheets and resemble a clam shell. The bound ligand is located within the interior of the "β-clam". Using the refined atomic coordinates of I-FABP and a sequence alignment of I-FABP and CRBP II (Jones et al., 1988), we have constructed a model of CRBP II (Li et al., 1989) to predict the side-chain orientations of its four tryptophan residues. The structure of CRBP II and its interaction with *all-trans*-retinol have also been probed with ^{19}F nuclear magnetic resonance (NMR) spectroscopy. In this study, 6-fluorotryptophan was used as the probe in F-labeled CRBP II and four mutant proteins constructed by genetic engineering (Li et al., 1989, 1990). In these mutant proteins, the W9F, W89F, W107I, and W110F mutants, each of four individual tryptophans was substituted by another hydrophobic amino acid. These studies indicate W107 to be at the ligand binding site.

Tryptophan fluorescence shifts appreciably to longer wavelength as the polarity of its environment increases (Mataga et al., 1964; Lumry & Hershberger, 1978), and its fluorescence lifetime varies with its conformation (Szabo & Rayner, 1980; Petrich et al., 1983). The tryptophan fluorescence of proteins may thus serve as a probe into the local protein structures surrounding tryptophans (Beecham & Brand, 1985; Creed, 1984). Since tryptophan in the nonpolar

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[1] Abbreviations: CRBP, cellular retinol binding protein; CRBP II, cellular retinol binding protein II; CRABP, cellular retinoic acid binding protein; I-FABP, rat intestinal fatty acid binding protein; L-FABP, rat liver fatty acid binding protein; one-letter abbreviations for amino acids, W, F, and I, are for tryptophan, phenylalanine, and isoleucine, respectively; W9F, W89F, W107I, and W110F represent mutant proteins in which tryptophans at a particular position have been substituted by another amino acid; EDTA, ethylenediaminetetraacetic acid.

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region of proteins emitting at a shorter wavelength may transfer its excitation energy to those in a more polar region emitting at a longer wavelength, time-resolved fluorescence spectroscopy may also probe into the long-range interaction between tryptophans in different environments of a protein. However, interpretation of the fluorescence of a multi-tryptophan protein, such as CRBP II, is often complicated, because it is often difficult to dissect the contributions of each individual tryptophan due to the overlap in their emissions, the contributions from each tryptophan in the total protein fluorescence may differ, and energy transfer may occur between them.

Recent development in the methods of molecular biology enables scientists to selectively replace individual tryptophans in a protein with another amino acid (Sommer et al., 1976; Brochon et al., 1977). A comparative fluorescence study of a specific protein containing two or more tryptophans and its mutant proteins in which individual tryptophans have been replaced by another amino acid will provide an additional probe for the structure of this protein. Such a technique has been applied recently to resolve the fluorescence of tryptophans in a number of proteins of known X-ray structures containing two to three tryptophans (Hansen et al., 1987; Hansen & Hillen, 1987; Harris & Hudson, 1990; Nishimura et al., 1990; Royer et al., 1990; Atkins et al., 1991; Smith et al., 1991).

The four tryptophans in CRBP II exhibit a composite fluorescence maximum at 337 nm which displays a complex decay pattern (Li et al., 1987). Our ability to overexpress CRBP II and the four genetically engineered mutant proteins in *E. coli* provided us with a useful model system for studying the fluorescence of multi-tryptophan proteins. Since each tryptophan was substituted by another hydrophobic amino acid which is nonfluorescent under our experimental conditions, the resulting changes in protein fluorescence offer the opportunity to obtain additional insights into the individual contribution of each tryptophan to protein fluorescence and about the local environment of each tryptophan.

This work deals with a fluorescence study of CRBP II mutant proteins. The binding efficiency of each protein to the ligand was determined. Subsequently, the fluorescence of each protein was analyzed by their lifetime at several wavelengths, and the quenching of their fluorescence by acrylamide was analyzed. The implications of these findings on the structure of CRBP II in conjunction with the ^{19}F NMR spectroscopy of its 6-fluorotryptophan analogues and molecular graphics (Li et al., 1989, 1990) will be discussed.

Materials and Methods

Materials. Plasmids and bacterial strains used for expression of rat CRBP II in *E. coli* have been described in earlier publications (Li et al., 1987, 1989). The construction, expression, and purification of site-directed mutant proteins have been reported in a recent publication (Li, 1990). Rat intestinal fatty acid binding protein, I-FABP, was expressed in and recovered from *E. coli* according to Sacchettini et al. (1990). *all-trans*-retinol was purchased from Kodak Laboratory and Specialty Chemicals (Rochester, NY). Acrylamide, EDTA, guanidine hydrochloride, and 2-mercaptoethanol were purchased from Aldrich, and acrylamide was recrystallized from ethyl acetate before use. All inorganic salts were reagent grade, and all solvents used were spectrograde. Doubly distilled water or HPLC grade water was used for all solutions.

Fluorescence Measurements. All experiments with *E. coli* derived proteins were performed in a 20 mM potassium phosphate buffer (pH = 7.4) containing 1 mM 2-mercaptoethanol (to prevent oxidation of cysteines in the protein), 1 mM EDTA, and 0.05% sodium azide. For most measurements, the protein concentration was adjusted to have an optical density of approximately 0.1 at the exciting wavelength. Steady-state measurements were carried out at 22 °C with a Perkin-Elmer MPF-66 spectrofluorometer with a constant-temperature cell-holder. Time-resolved fluorescence measurements were carried out at the same temperature with an instrument previously described (Chang et al., 1985). A bandpath of 2 nm was used for the excitation and emission slits. For time-resolved measurements, 500 channels of approximately 25 ps each were used to record the fluorescence decay, and 10 000 counts were stored for the initial fluorescence intensity.

Denatured protein solutions were prepared in 3 M guanidine hydrochloride.

Retinol Binding Study. Retinol binding studies were performed according to a known method (Cogan et al., 1976). The details were given in an earlier publication (Li et al., 1987). Two variations were used for the study, both of which yielded identical results. In the first, retinol was excited at 348 nm and the fluorescence monitored at 490 nm. In the second, the protein was excited at 290 nm, and the fluorescence at 340 nm was monitored. Since the binding ratio of protein to substrate is 1:1, the results were analyzed according to eq 1 where P_0 and R_0 are the protein and retinol concentrations,

$$P_0\alpha = R_0(\alpha/1 - \alpha) - K_d \quad (1)$$

respectively, α is the fraction of protein which exists as apoprotein at the given retinol concentration, and K_d is the apparent dissociation constant. Its value may be obtained from the intercept of a plot of $P_0\alpha$ vs $R_0(\alpha/1 - \alpha)$. We noted previously that binding of retinol to wild-type rat CRBP II decreases the native protein fluorescence by 90% (Li et al., 1987). However, the residual protein fluorescence in the mutant series after retinol binding varies with the point of substitution. The results are given in Table I.

Table I: Fluorescence and Ligand Binding Properties of I-FABP, CRBPs, and Mutant Proteins of CRBP II

protein/Trp	fluorescence max (nm)	fluorescence quantum yields[a]	K_d (nM)[b]	fluorescence of holoproteins (%)
CRBP	340	0.14	16[d]	
CRBP II	337	0.16	10 ± 6 10[e]	10
denatured CRBP and CRBP II	350	0.16		
W9F CRBP II	341	0.15	22 ± 8	10 ± 2
W89F CRBP II	341	0.17	40 ± 15	10 ± 2
W107I CRBP II	339	0.20	85 ± 19	33 ± 3
W110F CRBP II	327	0.13	64 ± 8	18 ± 2
I-FABP	328	0.26		
L-Trp	351	0.14[f]		

[a] Obtained with an excitation wavelength of 290 nm using L-tryptophan in water as the secondary standard. All values of proteins were determined in 0.02 M phosphate buffer, and denatured proteins in the same buffer plus 3 M guanidine hydrochloride. See Materials and Methods for a detailed description of the buffer. [b] K_d is the retinol binding constant. The values given are the average of two to four determinations. Uncertainties indicate the range of values from which the average was taken. [c] Residual tryptophan fluorescence at 340 nm relative to the apoprotein. [d] Literature value from Ong and Chytil (1978). [e] Literature value from MacDonald and Ong (1987). [f] Literature value from Eisinger (1969).

Quantum Yield Determination. The quantum yield of protein fluorescence was determined by using tryptophan in water as a secondary standard, $\Phi = 0.14$ (Eisinger, 1969).

Lifetime Measurements. The accuracy of the system was checked using an aqueous solution of *N*-acetyl-L-tryptophanamide. The excitation wavelength was 292 nm, and emission was monitored at several different wavelengths. The data were fit to a sum of exponentials:

$$K(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + A_3 \exp(-t/\tau_3) + \ldots \quad (2)$$

A more detailed description of the analysis is given in Cross and Fleming (1984).

Quenching Studies with Acrylamide. Protein concentrations ranging from 3.0 to 8.9 μM were used for the analysis. An excitation wavelength of 290 nm was used for all of the native proteins and 298 nm for the denatured CRBP II. The absorbance of the solution at the excitation wavelength was less than 0.1. Acrylamide was either added directly or added in microliter aliquots from a concentrated solution. Corrections were made for volume changes and attenuation of the excitation light by added acrylamide by using antilog $0.5A$ where A is the absorbance (Parker, 1968).

The Stern–Volmer equation (eq 3) was used to plot the acrylamide quenching of protein fluorescence (Eftink & Ghiron, 1976) where F_0 and F are the fluorescence intensities

$$F_0/F = 1 + K_{sv}[Q] \exp(V[Q]) \quad (3)$$

in the absence and presence of quencher, respectively, K_{sv} is the Stern–Volmer constant, which is equal to $k_q\tau_0$ where k_q is the rate constant for dynamic quenching, Q is the concentration of quencher, and V is the static quenching constant.

Results

Binding of Retinol to Apo-CRBP II and Its Mutant Proteins. The apparent dissociation constants, K_d, were measured for each tryptophan substitution mutant by fluorometric titration (see Materials and Methods), and the values are given in Table I. We found that the mutant proteins still bind retinol efficiently, although the binding efficiencies of mutant proteins are less than that of the wild type and vary over about 1 order of magnitude. The values are the average of two to four determinations. Uncertainties indicate the range of experimental values from which the average is taken. The uncertainties in the calculated values of K_d reflect the experimental method, because the intercepts of plots are very close to the origin with uncertainties approaching the values of the intercept (eq 1) (Li et al., 1987; Levin et al., 1988). However, the results indicate that substitution of W9 has the least effect on the binding efficiency while substitution of W107 causes the largest decrease in the binding efficiency.

Binding of retinol to wild-type CRBP II reduces native protein fluorescence to 10% of its original value (Li et al., 1987). The quenching is due to the energy transfer between the tryptophan residues and the bound retinol. Among the mutant proteins, only the W107I and W110F mutants show an appreciably less efficient retinol quenching than that in the wild type (Table I), and the quenching is least efficient in the W107I mutant, suggesting that W107 interacts most strongly with the bound retinol.

Fluorescence Emission of CRBP II and Its Mutant Proteins. Since the molecular model of CRBP II is based on the coordinates of I-FABP structure, the fluorescence of *E. coli* derived I-FABP was also measured for comparison. The fluorescence maximum of W110F mutant protein undergoes a large blue shift of 10 nm from that of the wild type to 327 nm, and approximates the fluorescence maximum of I-FABP (328 nm). Those of other mutant proteins undergo only a minor red shift. The emission maximum of wild-type CRBP and CRBP II under denaturing conditions was 350 nm, approximating that of L-tryptophan at 351 nm (Table I).

The fluorescence quantum yield increases appreciably only in the W107I protein (Table I). The quantum yields and

Table II: Calculated Fluorescence Maxima and Quantum Yields of Tryptophanyl Groups in CRBP II

group	fluorescence max (nm)	quantum yield
W9	331	0.19
W110	343	0.25
W89 + W107	328	0.10

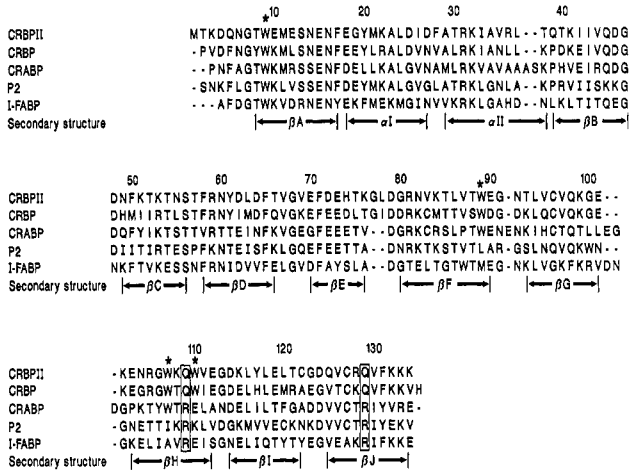


FIGURE 1: Alignment of three retinoid binding proteins with two fatty acid binding proteins with known X-ray structures, I-FABP and P2-myelin. Asterisks mark the positions of tryptophan in CRBP II. Regions of secondary structures in I-FABP and P2-myelin are given in the last row.

fluorescence spectra of the individual residues of CRBP II were estimated as follows. The quantum yield of CRBP II is given by

$$\Phi_{\text{CRBP II}} = (1/4)(\Phi_9 + \Phi_{89} + \Phi_{107} + \Phi_{110}) \quad (4)$$

Likewise, the quantum yields of the mutant proteins with one tryptophan replaced are given by eq 5 where Φ_x, Φ_y, and Φ_z

$$\Phi_{\text{mutant}} = (1/3)(\Phi_x + \Phi_y + \Phi_z) \quad (5)$$

refer to the quantum yields of the unsubstituted tryptophans. Implicit in the use of these equations was the assumption that each tryptophan residue had the same absorbance at the excitation wavelength. From the quantum yield of the W110F mutant, the combined quantum yields of W9, W89, and W107 can be determined from eq 5. This value can then be used in eq 4 along with the quantum yield of CRBP II to determine the quantum yield of W110. In a similar fashion, the quantum yield of W9 was also determined. From the estimated three-dimensional structure of CRBP II (Li et al., 1989), it was found that W89 may interact with both W107 and W110 (vide infra). Since W110 emits at the longest wavelength and will only accept energy from other W89 (Table II), it is reasonable to assume that its fluorescence behavior will not be appreciably influenced by this interaction. Due to the possibility of energy transfer between W107 and W89, their fluorescence behavior was grouped together and calculated in a similar fashion to those for W9 and W110.

The fluorescence spectra of the residues were obtained by assuming that the ratio of the fluorescence intensity of mutant proteins to that of native CRBP II at the same concentrations is given by eq 6 where the Φ's have the same meaning as

$$\text{ratio} = (\Phi_x + \Phi_y + \Phi_z)/\Phi_{\text{CRBP II}} \quad (6)$$

described previously. The spectra of W9 and W110 were obtained by adjusting the intensities of the native and mutant protein spectra accordingly and subtracting the mutant from the native spectrum. The spectra of CRBP II and its individual W groups are shown in Figure 2, and the quantum yields and

Table III: Fluorescence Lifetimes of I-FABP, CRBPs, and Mutant Proteins of CRBP II

protein[a]	wavelength monitored (nm)	A_1 (%)	τ_1 (ns)	A_2 (%)	τ_2 (ns)	A_3 (%)	τ_3 (ns)
CRBP[b]	340	43	4.43	28	1.57	29	0.18
CRBP II	315	24	3.48	46	0.89	30	0.10
	340	43	3.46	42	0.98	15	0.17
	350	53	3.96	32	1.01	15	0.18
denatured CRBP II	350	48	2.80	31	0.85	21	0.095
W9F CRBP II	318	32	3.07	35	0.61	35	0.085
	340	49	3.38	34	0.68	17	0.12
	370[c]	70	3.45	30	0.60		
W89F CRBP II	320	28	4.45	36	0.93	36	0.074
	370	48	6.39	30	1.05	22	0.083
W107I CRBP II	322	32	3.17	38	0.70	30	0.049
	340	49	3.70	37	0.81	14	0.12
	370[c]	82	3.96	18	0.82		
W110F CRBP II	315	49	1.76	26	0.62	24	0.078
	330	51	1.76	29	0.59	20	0.082
	360[c]	59	1.80	41	0.57		
I-FABP	328[c]	73	3.75	27	0.92		
	350[c]	74	3.95	26	1.14		

[a] Same solvents were used as those listed in Table I. The values are the average of two to four determinations, and the ranges of variation are less than 2%. Reduced χ^2 values were ≤ 1.20 in all cases. [b] Literature values (Levin et al., 1988). [c] Use of a triple-exponential decay function did not significantly improve the fit as judged by the χ^2 values.

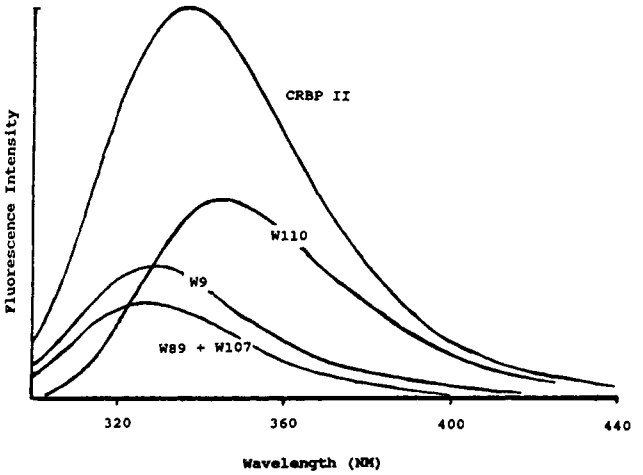


FIGURE 2: Calculated fluorescence intensities of tryptophan components in CRBP II on the basis of quantum yields of mutant proteins.

Table IV: Acrylamide Stern–Volmer Quenching Data for CRBP, CRBP II, and Mutant Proteins of CRBP II at 25 °C

protein	K_{sv} (M^{-1})	V (M^{-1})	τ (ns)[a]	calcd τ (ns)[b]	k_q ($\times 10^9$ M^{-1} s^{-1})[c]
CRBP[d]	5.3	≈0	2.4		2.2
CRBP II[e]	3.8	≈0	1.9	1.82	2.0
denatured CRBP and CRBP II	6.5	1.0	1.6		4.9[f]
W9F CRBP II	4.9	0.4	1.9	1.73	2.6
W89F CRBP II	5.7	≈0	2.5	2.55	2.3
W107I CRBP II	6.9	≈0	2.1	2.08	3.3
W110F CRBP II	2.3	≈0	1.1	1.15	2.1

[a] Average lifetime calculated from data in Table III. Lifetimes were taken from the data nearest to the maximum of emission except for W89F CRBP II, for which the lifetime was taken from the average of the lifetimes at 320 and 370 nm. [b] Values calculated from Scheme I. [c] Excitation wavelength, 290 nm. [d] Literature values (Levin et al., 1988). [e] Quenching of CRBP II was done at 20 °C. [f] Corrected for the increase in viscosity due to guanidine hydrochloride by multiplying K_{sv} by a factor of 1.2 (Kawahara & Tanford, 1966); excitation wavelength, 298 nm.

approximate fluorescence maxima of the W groups are listed in Table II.

Time-Resolved Fluorescence Study of CRBP II and Its Mutant Proteins. The fluorescence decays of CRBP II and its mutant proteins were examined at several different wavelengths ranging from 315 to 370 nm. The fluorescence decay of I-FABP was examined also for comparison, and the results are tabulated in Table III. The fluorescence decay of I-FABP may be expressed as a double exponential and is relatively wavelength independent indicating the environments of the two tryptophans are relatively homogeneous. The fluorescence decays of CRBP II and its mutant proteins are usually best expressed in three exponents ranging from approximately from 0.1 ns to several nanoseconds. Since the environments of tryptophans in these proteins are apparently different, giving rise to different spectra, it is reasonable to expect energy transfer to occur among them. This is in accord with the experimental observation that the contribution by the longest lifetime component in the total fluorescence, α_1, always increases as the wavelength of analysis increases. Interestingly, when the lifetimes of mutant proteins are examined at the red edge of the fluorescence (350–370 nm), the decay patterns of W9F, W107I, and W110F mutant proteins simplify to a double exponential, while that of W89F does not. The lifetime of the W89F protein shows the largest increase from that of the wild type, while that of the W110F protein shows the largest decrease.

Stern–Volmer Quenching Analysis of CRBP, CRBP II, and CRBP II Mutant Proteins. The solvent accessibilities of tryptophan residues in these proteins were also probed by acrylamide quenching experiments, and the results are shown in Table IV. The quenching rates, k_q, were calculated from the Stern–Volmer constants and the average lifetimes derived from the time-resolved fluorescence studies (Table III). Upon denaturation of native CRBP and CRBP II, nonlinear Stern–Volmer plots were obtained, and the k_q increases to 4.9 $\times 10^9$ M^{-1} s^{-1} with a static quenching constant (V) of 1.0 M^{-1}, corresponding to values of unshielded tryptophans in proteins (Eftink & Ghiron, 1977). Among all mutant proteins, although there is a general trend in increasing k_q over the wild-type protein, only the W9F mutant protein exhibits a tendency to undergo static quenching (Figure 3).

Discussion

The structures of fluoro analogues of CRBP II and its mutant proteins in which the tryptophans have been substituted by 6-fluorotryptophan, both in their holo and in their apo forms, have been studied by ^{19}F NMR spectroscopy (Li et al., 1989, 1990). Four mutant proteins were constructed in which individual tryptophans were substituted by another hydro-

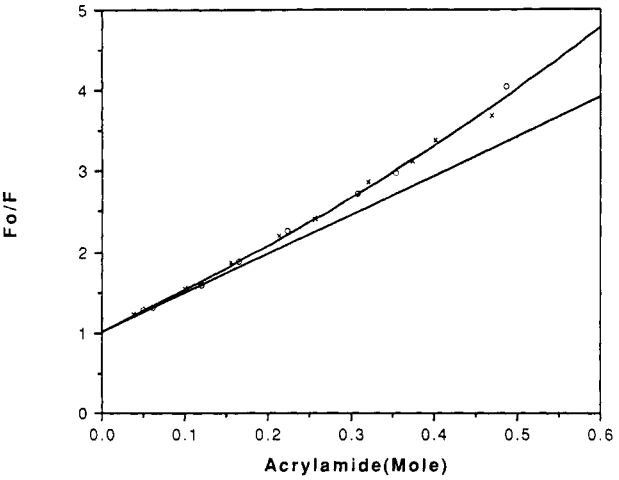


FIGURE 3: Stern–Volmer plot of the fluorescence quenching of the W9F mutant protein by acrylamide. The straight line represents the theoretical data if no static quenching is involved. The top line is the experimental data, and (×) and (○) represent the results of two different determinations.

phobic amino acid. Among them, W9, W89, and W110 were substituted by a phenylalanine (F), since F for W substitutions have been shown previously to preserve the overall conformation of a number of proteins (Rule et al., 1987; Hansen et al., 1987), and W107 was replaced by isoleucine (I), since sequence alignments indicate that I107 (CRBP II numbering) of the P2-myelin (Figure 1) also binds *all-trans*-retinol in vitro (Uyemura et al., 1984). The results clearly demonstrate that W107 plays an important role in the binding of retinol. The changes in the ^{19}F resonances of all tryptophans other than W107 in 6-fluorotryptophan-labeled apo-CRBP II are small. However, W9 and W107 resonances in the ^{19}F NMR spectrum of the fluorinated W89F mutant protein show a more complicated set of peaks than that of wild-type CRBP II. This suggests that W9 and/or W107 in W89F apo-CRBP II may exist in more than one conformation on the NMR time scale and may have a greater degree of freedom. The W9 resonance in the W107I mutant compound is broadened and may also reflect a change in the dynamics of this residue. The W9 resonance is shifted 0.9 ppm downfield in the W110F mutant compound compared to the wild-type compound, suggesting that there is a change in the local environment of W9 when W110 is substituted by a phenylalanine. In order to probe further into its structure, CRBP II and its four mutants proteins are probed by fluorescence spectroscopy. The four mutant proteins used are the same as those used in our NMR study (Li et al., 1990). The fluorescence of these mutant proteins was investigated with respect to their wavelength, ligand binding efficiency, lifetime at several different wavelengths, and quenching with acrylamide. This information, coupled with that from our NMR studies and molecular graphics (Li et al., 1989, 1990), will be applied to gain insight into the structure and function of CRBP II.

Structural Relationship between CRBP II and I-FABP. A three-dimensional diagram for CRBP II has been constructed from the known structures of I-FABP and P2-myelin (Sacchettini et al., 1988, 1989a,b; Jones et al., 1988) on the basis of their similar molecular sizes and their amino acid homology, particularly between the N-terminal up to about position 100 (CRBP numbering) (Li et al., 1989). It is reasonable to project that CRBP II, like I-FABP and P2-myelin, may also possess a β-clam structure. A comparative study on the fluorescence between I-FABP and CRBP II was thus made.

It should be noted, however, there is no amino acid analogy between CRBP II and I-FABP in positions 100–112, βH-region, where two of the four tryptophans, W107 and W110, are located (Figure 1). It is probable that the structure of CRBP II may differ somewhat from the known structure of I-FABP in that region. A fluorescence study may provide us with the experimental evidence to resolve this question. It may be noted also that W107 and W110 are present in both retinol binding proteins but are absent in all fatty acid binding proteins and W110 is absent in the cellular retinoic acid binding protein CRABP (Sundelin et al., 1985). However, all fatty acid binding proteins and CRABP, except L-FABP (Sacchettini et al., 1990), contain an arginine, R, at positions 109 and 129 which are absent in CRBP and CRBP II, neither of which binds retinoic acid (Levin et al., 1988).

Rat intestinal fatty acid binding protein, I-FABP, contains two tryptophans in positions equivalent to 9 and 87 in CRBP II (Figure 1). Since positions 87 and 89 are both in the βF-strand part of the protein structure and hydrophobic (or hydrophilic) side chains of amino acids tend to occupy alternative positions in a β-strand resulting in two amphiphilic faces (Kaiser & Kezdy, 1984), W89 in CRBP II and W87 in I-FABP are likely in a similar environment. The fluorescence maximum of I-FABP is at a very short wavelength of 328 nm, suggesting that both tryptophans are shielded from the solvent. The fluorescence lifetime of I-FABP may be expressed simply as a double exponential and is relatively wavelength independent. All these results are consistent with the X-ray structure in that the two tryptophans are situated relatively far apart on the hydrophobic side of a β-clam structure.

The fluorescence maxima of both CRBPs, 337 and 340 nm, are at a longer wavelength than I-FABP, and shift to an even longer wavelength, 350 nm, upon denaturation. The fluorescence decays of both CRBPs are more complex than that of I-FABP and may be fitted in a three-component exponential. When the lifetime of CRBP II is monitored at three different wavelengths from 315 to 350 nm, the longest lifetime component increases both in duration and in amplitude, from 3.48 to 3.96 ns and from 24% to 53%. The results suggest a more inhomogeneous environment among its four tryptophans with one or more of the tryptophans situated in a more polar environment than those in I-FABP. Very probably, singlet energy transfer may also occur among the tryptophans if they are closer together than those in I-FABP. Such a process may further contribute to the complexity of fluorescence decay patterns of CRBP II and its analogues.

Retinol Binding of CRBP II Mutant Proteins. The measured apparent dissociation constants for retinol binding, K_d, increase in the order W9F, W89F, W110F, and W107I. The results indicate that, although mutant proteins still bind retinol efficiently, substitution of W107 by isoleucine decreases the binding affinity by approximately 1 order of magnitude (Table I). This is not surprising since W107 is present in all retinoid binding proteins and not in fatty acid binding proteins (Figure 1) and it is most affected by retinol in the holoprotein in our NMR study (Li et al., 1989, 1990). It is interesting, however, that substitution of a single tryptophan away from the binding site may also decrease the binding affinity by a factor of approximately 2–6, suggesting that there may be a general overall change in the protein structure.

Binding of retinol to wild-type CRBP II reduces the native protein fluorescence by a factor of 10 (Table I). The quenching is due to energy transfer between the tryptophans and the bound ligand. The reduction is about the same for the W9F and W89F mutant proteins since both W9 and W89

are apparently located away from the binding site. The reduction of the W107I mutant protein fluorescence by bound retinol is only by a factor of 3, and the observation suggests that W107 interacts most strongly with the ligand. The efficiency of energy transfer from excited tryptophans to bound retinol in the W110F protein is also less than that in the wild-type CRBP II. This decrease may be related to the decreased fluorescence quantum yield and shortened lifetime of this apoprotein (Tables I and II).

Fluorescence Maxima and Quantum Yields of CRBP II Mutant Proteins. The fluorescence maximum of the W110F mutants exhibits a dramatic 10-nm shift to the blue from that of wild-type CRBP II. This suggests that W110 is located in a polar environment since replacement of a residue in a polar environment should result in a blue shift providing that the other three tryptophans are in less polar environments. The observation that the ^{19}F resonance of 6-F-W110 is upfield from other three residues is also consistent with this residue being in a more polar environment (Li et al., 1989, 1990).

A CRBP II structure constructed from molecular graphics (Li et al., 1989) based on the known structures of I-FABP and P2-myelin (Sacchettini et al., 1989a,b; Jones et al., 1988) suggests that W110, which corresponds to Q107 of I-FABP, will be located at the end of the βH-strand pointing *away* from the β-clam. It also predicts that the side chain of W110 is partially shielded from the solvent by a number of polar groups including R92, T93, K108, and E112. The polarity of the environment surrounding W110 may be in part be due to the proximity of these polar side chains rather than entirely to its exposure to the aqueous solvent. Our experimental data from fluorescence and fluorescence quenching by acrylamide are in good agreement with the result from molecular graphics.

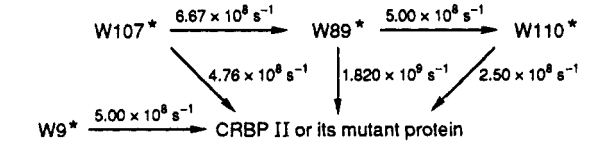
The minor shifts in protein fluorescence to the red by 2–4 nm in other mutant proteins suggest that other tryptophans, W9, W89, and W107, are situated in relatively hydrophobic environments (Table I).

The fluorescence quantum yield of CRBP II, 0.16, is not appreciably affected by denaturation. However, it is decreased in the W110F mutant protein, suggesting that W110 emits with the highest quantum efficiency among the tryptophans in CRBP II (Table II).

Fluorescence Lifetime Studies of CRBP II Mutant Proteins. We have noted that CRBP II exhibits a more complex fluorescence decay pattern than that of I-FABP. Since W110 has been shown to be in a more polar environment than the other tryptophans, fluorescence lifetimes of mutant proteins were monitored near the red edge to explore its behavior and examined at several wavelengths in order to explore the possibility of energy transfer from the "blue-emitting" tryptophans to W110 (Table III). Three noteworthy observations were made.

(a) At the red edge, the fluorescence decay pattern of all mutant proteins except W89F simplifies, to a double exponential (Table III). This difference may be attributed to modification of the environment surrounding W110. The W89F substitution may cause a change in protein conformation which imparts greater motional freedom in W110. Complex decay patterns have been observed from a single tryptophan in proteins and have been interpreted in terms of interconversion between a large number of conformations, each with a distinct lifetime (Alcala et al., 1987a,b). If the rate of interconversion between conformations becomes comparable to the rate of fluorescence, the exponentiality of decay becomes more complex. Such an explanation is in agreement with our observation on the ^{19}F NMR spectrum of the 6-fluorotryptophan analogue of this mutant apoprotein. Its ^{19}F NMR spectrum shows a more complicated set of peaks in the region of W9 and W107 resonance than exists in the corresponding fluorinated CRBP II and other mutant proteins (Li et al., 1990). This suggests that W9 and/or W107 in the apoprotein can exist in more than one conformation on the NMR timescale and can have a greater degree of freedom. Such freedom disappears in the holoprotein.

Scheme I



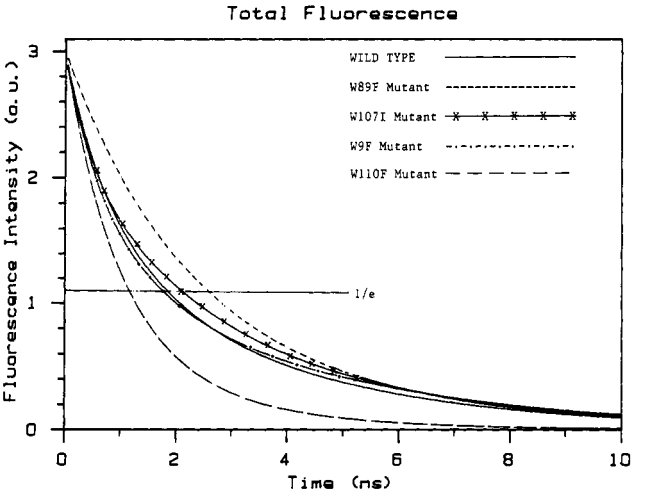


FIGURE 4: Calculated average fluorescence lifetimes of CRBP II and its mutant proteins on the basis of fluorescence quantum yields listed in Table II and rates listed in Scheme I.

(b) The W89F protein displays the longest average lifetime (2.5 ns) as well as the longest lifetime of its lengthiest component among the wild-type and mutant proteins. These observations may be modeled by assuming that W89 plays a significant role in the transfer of excitation energy to W110. On the basis of the lack of interaction between W9 and W87 (CRBP II numbering) in I-FABP and its known structure, we have assumed W9 to be an independent emitter with a lifetime of 2 ns. Using the value of the fluorescence quantum yield, Φ_f, of 0.19 from Table II, its calculated radiative lifetime, τ_f/Φ_f or $(k_f\Phi_f)^{-1}$, is thus 10.5 ns. Since W107 and W110 are situated at the opposite side of a β-strand, it is also reasonable to assume that they are at a noninteracting distance. However, W107 may still transfer its energy to W110 via W89. To explore this idea, we have set up the simple model shown below in Scheme I (Jean et al., 1988). Using the radiative lifetime of W9 as the standard for all tryptophans in CRBP II, a set of simultaneous equations to describe the behavior of individual tryptophans may be set up by using the quantum yields listed in Table II and the lifetimes listed in Table IV. A set of rate constants may thus be calculated by solving these equations, and the results are given in Scheme I. The calculated lifetimes for CRBP II and its mutant proteins at 330 nm are given in Figure 4 and Table IV. The calculated average lifetimes are in excellent agreement with the experimental values.

The singlet energy transfer between tryptophans in proteins has been analyzed previously by Eisinger (Eisinger et al., 1969) using the Förster theory (Förster, 1948, 1949, 1959). The Förster critical distance for the transfer between tryptophans, R_0, the distance at which the rate of energy transfer is equal

to the sum of the rates of all other modes of deexcitation of the donor, is given by eq 7 where Φ_D is the fluorescence

$$R_0^6 = (8.8 \times 10^{-25})\Phi_D\kappa^2 n^{-4} J_{AD} = (6.1 \times 10^{-43})\kappa^2 \quad (7)$$

quantum yield of the donor, κ is the orientation factor of interaction between the donor and the acceptor, n is the refractive index of the medium, and J_{AD} is an overlap integral. The size of the overlap integral will depend on the fluorescence wavelength of the donor tryptophan. Those in the hydrophobic environment emitting at a shorter wavelength, <330 nm, such as W107 and W89, will be more efficient donors than W110. Assuming the fluorescence maximum of a tryptophan to be 330 nm and the quantum yield to be 0.15, we can obtain values of R_0 for different values of the orientation factor, κ. For values of κ^2 ranging from $^2/_3$ for a random orientation to 4 for an optimum alignment, the value of R_0 may vary from 8.6 to 11.6 Å, which is in the range of R_0 predicted by molecular graphic analysis of our model of CRBP II (J. Sacchettini, personal communication). Since the values of Φ_D, κ, n, and J_{AD} may all vary from a particular tryptophan in one protein to another, and the three-dimensional structure of CRBP II used in our study is constructed from molecular graphics, these limitations render a more accurate evaluation of the energy transfer between tryptophans in CRBP II difficult. Using the estimated lifetime of W9 in CRBP II or the experimental value of tryptophans in I-FABP as the lifetime of a typical tryptophan in a hydrophobic environment, we obtain a rate of energy transfer over a distance of 10 Å to be (2.0–9.1) × 10^8 s^{-1} for values of κ^2 ranging from $^2/_3$ to 4, which is in general agreement with the values of the calculated rates of (5.0–6.67) × 10^8 s^{-1} found in Scheme I.

Therefore, the changes in the fluorescence lifetime in the four mutant proteins of CRBP II can be modeled as shown in Scheme I. In particular, the role of W89 in mediating the transfer from W107 to W110 seems significant. Our data suggest a rather wide range of lifetimes for the individual tryptophans with W110 being the longest lived and W89 the shortest.

(c) The analysis of the fluorescence lifetime of the W110F mutant protein at 315, 330, and 360 nm indicates that there is no change within experimental error in the lifetime and a small change in the magnitude of the longest component with the detection wavelength. The result suggests that the environments of its three interior tryptophans, W9, W89, and W107, as in the case of I-FABP, are relatively homogeneous. The excited-state interaction between W89 and W107 may have shortened its fluorescence lifetime.

Quenching of Fluorescence of CRBP II Mutant Proteins by Acrylamide. Stern–Volmer analysis of the quenching of fluorescence of tryptophan and its derivatives by acrylamide has been studied by Eftink and Ghiron (1976). The plots show a distinct upward curvature at higher quencher concentration with a rate higher than the diffusion-controlled process from the static quenching. Proteins containing nonsurfacial tryptophan are quenched by acrylamide with a rate less than a diffusion-controlled process (Eftink & Ghiron, 1977). Acrylamide quenching may thus serve as a measure of solvent exposure of tryptophan in proteins. Previously we have shown that native CRBP and CRBP II undergo acrylamide quenching with linear Stern–Volmer plots and a rate less than a diffusion-controlled process, indicating that tryptophans in these proteins are not directly accessible to the quencher. The quenching rates increased and static quenchings occurred after the proteins were denatured (Table IV). When this technique was applied to mutant proteins of CRBP II, we found that all mutant proteins were quenched by acrylamide with a rate less than a diffusion-controlled process. The fluorescence of W107I protein was quenched most efficiently, suggesting that W107 is the least accessible of the four tryptophans in CRBP II, while the fluorescence of the W110F mutant protein was quenched with the lowest efficiency, suggesting that W110 is the only tryptophan located in a polar environment partially exposed to the solvent. Although the W9F mutant protein undergoes acrylamide quenching with a rate comparable to other mutant proteins, it undergoes static quenching (Table IV and Figure 3). When our results are examined as a whole, it is concluded that W110, being on the opposite side of W107 in the β-clam pointing away from the binding pocket, is the most exposed among the four tryptophans. However, W110 in CRBP II and its mutant proteins other than the W9F mutant does not undergo static quenching. This result suggests that W110 in CRBP II is only partially exposed to the solvent and the W9F mutant protein may have a looser protein structure and greater solvent and quencher accessibility to at least one of its tryptophans, most likely W110. This suggestion is in agreement with our observations on the ^{19}F NMR spectra of 6-fluorotryptophan-labeled apo-CRBP II and its W9F mutant protein that W110 in the mutant protein may be in a less restricted environment (Li et al., 1989).

Summary and Conclusions

Our fluorescence investigation on CRBP II and its four single mutant proteins in which four tryptophans have been replaced individually by another hydrophobic amino acid provides additional support to the conclusions of an NMR study and a molecular graphic analysis that CRBP II possesses a similar overall structure of those of I-FABP and P2-myelin. Three of the four tryptophans, W9, W89, and W107, are in the hydrophobic region of the protein, while W110 is more exposed to the solvent and surrounded by polar amino acids. Tryptophan in position 107 is in the ligand binding pocket and plays an important role in ligand binding.

Ideally, conservative mutation of this kind would have no effect on the protein structure, and properties of mutant proteins would closely parallel those of the native protein. A careful examination on the properties of these mutant proteins and its 6-fluorotryptophan-labeled analogue suggests that this ideal is not met. Although W9 is furthest from the ligand binding site, substitution of W9 by phenylalanine leads to the static quenching of the mutant protein fluorescence by acrylamide, suggesting that W9 has a role in the overall folding of the protein. Time-resolved fluorescence study of the mutant proteins reveals that W89 mediates the energy transfer between W107 and W110. Although the interior W89 and the exterior W110 are remote from the ligand binding site, they have important structural functions in ligand binding. A conservative mutation of either of these two positions to another aromatic amino acid, phenylalanine, loosens the protein structure and lowers its ligand binding efficiency appreciably. Since these effects may be cumulative on the protein structure and function, the results of this study suggest that double and higher mutants of this family of proteins would have an appreciably different structure from the native protein. Any conclusions derived from higher mutants in this family of proteins must be treated with caution.

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