Test of the Contribution of an Amino−Aromatic Hydrogen Bond to Protein Function†

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ABSTRACT: Hydrogen bonds which form between a hydrogen bond donor and an aromatic ring as acceptor are thought to contribute to the stability and function of proteins. We have tested the function of such an interaction in a highly homologous pair of proteins, cellular retinol-binding protein (CRBP) and cellular retinol-binding protein, type II [CRBP(II)]. Both proteins bind the ligand *all-trans*-retinal with comparable affinities, but CRBP has an approximately 100-fold higher affinity for *all-trans*-retinol. The greater affinity of CRBP for *all-trans*-retinol has been attributed to the presence of an amino−aromatic hydrogen bond, which is absent in CRBP(II). We have generated a pair of mutant proteins, in which the amino−aromatic interaction was removed from CRBP and introduced into CRBP(II). Spectral analyses of retinol when bound to the wild-type and mutant CRBP suggested that it adopted an identical conformation within both proteins, a conformation that was distinct from that of retinol bound to CRBP(II), both wild-type and mutant. Unexpectedly, the affinities of the mutant binding proteins for *all-trans*-retinol were indistinguishable from those of their corresponding wild-type proteins. Further, in ligand competition experiments, there were no observable differences between mutant and wild-type CRBP, or between mutant and wild-type CRBP(II), in their preferences for binding *all-trans*-retinol versus *all-trans*-retinal. The results of this direct test of the proposed function of an amino−aromatic hydrogen bond did not support a functional role for such bonds, at least in this system.

"Unconventional hydrogen bonds" formed between a hydrogen bond donor and an aromatic ring acceptor have been described in model systems where benzene serves as a hydrogen bond acceptor for water (Suzuki et al., 1992) and ammonia (Rodham et al., 1993). It has been suggested that such bonds may provide structural stability and/or have a functional role in proteins. Possible contributions of amino−aromatic hydrogen bonds to function have been described in a variety of systems, one of which is phosphotyrosine recognition by the SH2 domain of v-*src* (Waksman et al., 1992). The evidence for or against amino−aromatic hydrogen bonds making a significant contribution to the stability or function of a protein structure has, to date, been computational and statistical. Better evidence might be provided by a direct experimental test.

Cellular retinol binding protein (CRBP,[1] referred to here as type I) and cellular retinol-binding protein, type II, provide a naturally-occurring system for such a test. These proteins have 56% sequence identity, and the traces of their polypeptide backbones are virtually superimposable (Banaszak et al., 1994). Both bind retinol and retinal, forming a single protein−ligand hydrogen bond with a conserved amino acid (Gln-108). The two proteins have similar affinities for retinal. However, the affinity of type I for retinol is estimated to be 100-fold higher than its affinity for retinal and similarly greater than the affinity of the type II protein for retinol (Li et al., 1991). The affinity of type II for retinol is only 3-fold better than it is for retinal (Li et al., 1991; Dew & Ong, 1994). The explanation tendered for these facts (Banaszak et al., 1994; Cowan et al., 1993) has been that Gln-108, which hydrogen bonds to the retinol hydroxyl in both holoproteins (Figure 1), also makes an amino−aromatic interaction with Phe-4 in type I. In type II, the Phe-4 is replaced by a Gln residue.

In type I, the amino group of Gln-108 is 3.85 Å from the ring centroid of Phe-4 (Cowan et al., 1993). Levitt and Perutz calculated the energy minimum to be at 3.4 Å for an amino−aromatic hydrogen bond, the distance found in the ammonia/water−benzene model systems, but even at 3.85 Å their calculated contribution to stability is greater than 2.5 kcal (Levitt & Perutz, 1988). This 2.5 kcal could readily account for the 2 orders of magnitude difference in K_d of the type I protein for retinol and retinal. Since the NH of the side chain amide of Gln-108 is oriented to serve as a proton donor to the phenylalanine ring, it must be the carbonyl part of the side chain amide which hydrogen bonds to the retinol (Figure 1). Consequently, Gln-108 is restricted to serve as a H-bond acceptor for the ligand. Should Gln-108 need to serve as a H-bond donor, as it would if the ligand were the aldehyde retinal, the favorable amino−aromatic interaction would be lost (Banaszak et al., 1994; Cowan et al., 1993). It is postulated that this amino−aromatic hydrogen bond allows the type I protein to discriminate between the alcohol and aldehyde ligand. Because the amino acid at position 4 is a Gln for type II, the Gln at position 108 can orient as either a hydrogen bond donor or a hydrogen bond acceptor, without the consequent loss of any other favorable interaction. This would explain why type II binds the aldehyde and the alcohol with similar affinities.

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[1] Abbreviations: CRBP, cellular retinol-binding protein; F4Q-CRBP, CRBP with a Phe to Gln substitution at residue 4; CRBP(II), cellular retinol-binding protein, type II; Q4F-CRBP(II), CRBP(II) with a Gln to Phe substitution at residue 4.

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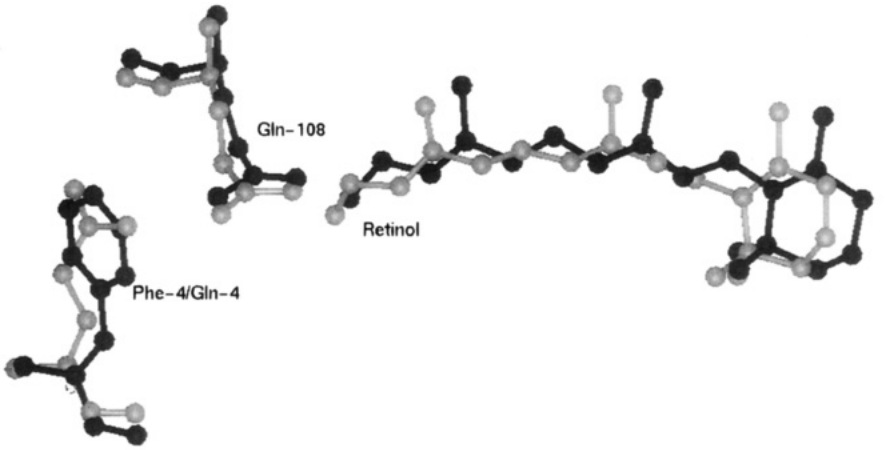

FIGURE 1: Gln-108 hydrogen bonds to the retinol hydroxyl in both type I (black) and type II (gray) cellular retinol-binding proteins. In type I, Gln-108 also participates in an amino−aromatic hydrogen bond.

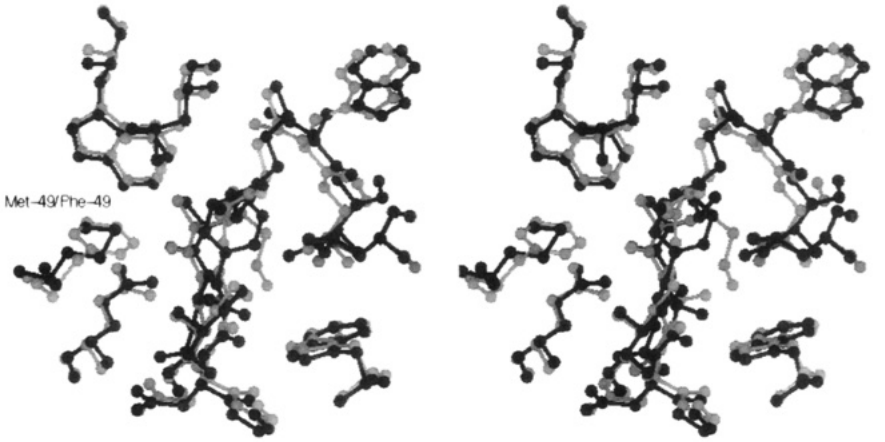

FIGURE 2: Stereoview of the context in which amino acid position 4 (center) is located in type I (black) and type II (gray).

As noted above, the structures of type I and type II, both of which have been determined to a resolution of 2 Å (Cowan et al., 1993; Winter et al., 1993), are highly conserved. The part of the small hydrophobic core of the protein, where the Gln-4/Phe-4 is located, is also highly conserved. Comparison of the structures of the homologous proteins around amino acid position 4 revealed no obvious reason why a Phe placed at position 4 in the type II protein would not be able to adopt the same conformation as Phe-4 in type I, and provide an aromatic ring with which Gln-108 could interact. Likewise, a Phe→Gln mutation should also be readily accommodated in CRBP. Of the 15 side chains within ~5 Å of position 4, 11 are identical in the 2 proteins and adopt equivalent conformations (Figure 2). Only one of the four changes (Met-49 in type I is a Phe in type II) is in a buried amino acid side chain, in the direct vicinity of position 4. The other three substitutions are in surface side chains. If an amino−aromatic interaction between Phe-4/Gln-108 is involved in CRBP I function, then replacement of Phe-4 with a Gln should reduce its affinity for retinol and its ability to discriminate between retinol and retinal. Conversely, replacement of Gln-4 with a Phe in type II could create a type II protein that has increased affinity for retinol and now discriminates more strongly between retinol and retinal than does the wild-type protein. Thus, this pair of proteins provided us with a well-characterized context in which to make single-site mutations that we knew could be accommodated easily and would test the importance of the amino−aromatic bond in CRBP function.

EXPERIMENTAL PROCEDURES

Materials. Using a 5′ primer which encoded the desired mutation, mutant binding protein coding sequences were amplified by PCR. For generation of mutant type I (F4Q-type I) coding sequence, the following 5′ primer was used: 5′-GGGGGGCATATGCCTGTGGACCAGAACGGGTAC-TGGAAGATGCTGAGC-3′. A 3′ primer was used which introduced a *Cla*I site 3′ to the type I coding sequence: 5′-GGGGGGATCGATTCAGTGTACTTTCTTGAACACTT-GCTTGCAGG-3′. For generation of mutant type II (Q4F-type II) coding sequence, the following 5′ primer was used: 5′-CCCCATATGACGAAGGACTTCAATGGAACCTGG-3′. A 3′ primer was used which introduced a *Hin*dIII site 3′ to the type II coding sequence: 5′-CATGTTCCAAGCT-TCCCCTGGGCCC-3′. The rat cDNAs for either type I (Sherman et al., 1987) or type II (Li et al., 1986) were used as templates. Following their confirmation by dideoxy-nucleotide sequencing, the mutant protein coding sequences were cloned into the bacterial expression vector pT7 (Tabor & Richardson, 1985) at the *Nde*I/*Cla*I sites for F4Q-type I and at the *Nde*I/*Hin*dIII sites for Q4F-type II. Mutant proteins were expressed in log-phase BL21-DE3/pLYS-S cells (Stratagene) by induction with 0.5 mM IPTG for 3 h at 37 °C with shaking. The mutant proteins were purified from bacterial lysates by methods described previously (Herr & Ong, 1992; Dew et al., 1993).

Competition between all-trans-Retinol and all-trans-Retinal for Protein Binding. Unless otherwise indicated, all

procedures involving retinoids were performed using a yellow safe light for illumination. Apo forms of the binding proteins (1 μM final in 500 μL of 10 mM Tris−acetate, pH 8.3) were individually incubated overnight at 4 °C in the dark with [^{3}H]-*all-trans*-retinol (1.2 μM final) and increasing amounts of cold competitor. Before their addition, the retinoids were mixed, dried under a stream of nitrogen, and resuspended in 5 μL of DMSO. After the overnight incubation, 400 μL of the reaction mixture was loaded on a 0.5 mL DE-52 column (Whatman) equilibrated in 10 mM Tris−acetate, pH 8.3. The column was washed 3 times with 2 mL of equilibrating buffer, and protein was eluted with 2 mL of 0.33 M Tris−acetate, pH 8.3, into 100 μL of a 10 mg mL^{-1} BSA solution; 200 μL of the eluate was mixed with 2 volumes of ethanol before counting. Background was determined by counting the reaction mixture in the absence of binding protein.

Determination of Relative Affinities of Wild-Type and Mutant Binding Proteins for all-trans-Retinol. For comparison of relative affinities of wild-type and mutant CRBP, either CRBP or F4Q-CRBP at a final concentration of 1.5 μM and CRBP(II) at a final concentration of 22.5 μM were mixed with 0.3 μM [^{3}H]-*all-trans*-retinol (final) in 500 μL of 50 mM imidazole acetate, pH 6.4. Because of its lower affinity for retinol, the use of a large molar excess of CRBP-(II) was necessary for measurable levels of competition. The mixture was incubated at 4 °C overnight in the dark, following which 270 μL of the reaction mixture was loaded onto a 1.2 mL DE-52 column (Whatman) preequilibrated in 50 mM imidazole acetate, pH 6.4, and CRBP(II) was eluted from the column with 2 mL of 50 mM imidazole acetate, pH 6.4, while CRBP and F4Q-CRBP were eluted with 2 mL of 200 mM imidazole acetate, pH 6.4. Four hundred microliters of each of the two fractions was added to 800 μL of ethanol, and radioactivity was determined by scintillation counting as described above.

For determination of the relative affinities of CRBP(II) and Q4F-CRBP(II) for *all-trans*-retinol, CRBP at a final concentration of 0.5 μM and CRBP(II) or Q4F-CRBP(II) at a final concentration of 5 μM were mixed with 0.3 μM (final) [^{3}H]-*all-trans*-retinol in 500 μL of 50 mM imidazole acetate, pH 6.4, 20 mg/mL BSA. The mixture was incubated overnight at 4 °C in the dark, and the proteins were separated by DE-52 chromatography as described above. The radioactivity of the two fractions was determined as described above.

RESULTS AND DISCUSSION

Absorbance Spectra of Holo Mutant Binding Proteins. After the generation of the mutant proteins F4Q-type I and Q4F-type II, an initial test of ligand binding characteristics was the determination of the absorbance spectra of the mutant proteins in the presence of *all-trans*-retinol. The distinctive absorption spectra of retinol (Ong, 1984) when complexed with CRBP and CRBP(II) are a highly sensitive indicator of chromophore conformation. The spectra of retinol bound to the mutant proteins were superimposable upon the spectra of retinol when bound to the respective wild-type proteins (Figure 3), indicating the retinol environments were not altered in the two mutants. In addition, the 2 nm difference in λ_{max} of the retinol spectra between type I (350 nm) and type II (348 nm) was preserved in the mutant complexes.

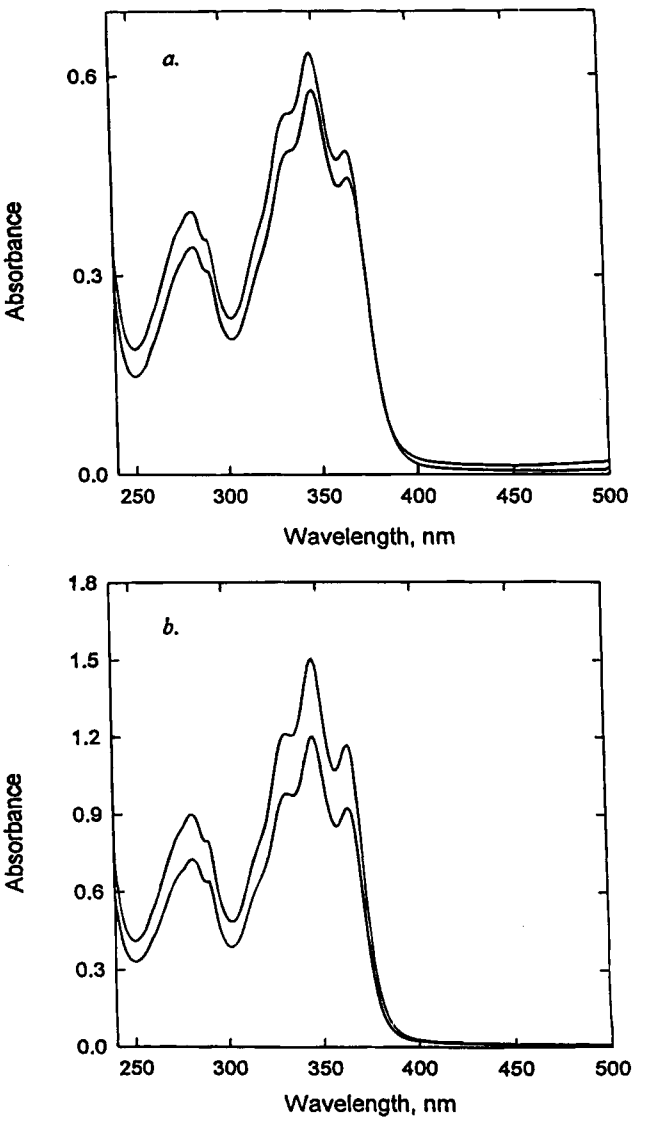

FIGURE 3: Absorbance spectra of *all-trans*-retinol complexed with (a) F4Q-mutant (top) and wild-type (bottom) CRBP, and (b) Q4F-mutant (top) and wild-type (bottom) CRBP(II).

Competition between all-trans-Retinol and all-trans-Retinal for Protein Binding. To test the specificity of the type I mutant, we compared the ability of unlabeled retinal to compete for the binding of [^{3}H]retinol. Wild-type or mutant binding proteins were mixed with a 1.2-fold molar excess of [^{3}H]-*all-trans*-retinol and increasing amounts of unlabeled *all-trans*-retinal. The mixture was incubated overnight at 4 °C, a time sufficient to reach equilibrium, based on our determination of off rates (Rexer and Ong, unpublished results). Following this, protein and unbound ligand were separated by ion exchange chromatography, and the amount of bound [^{3}H]retinol was determined by scintillation counting. At an 8-fold molar excess of unlabeled retinal, the modest reduction in binding of labeled retinol observed for the mutant was no greater than that observed for the wild-type protein (Figure 4a). Competition with unlabeled retinol served as a control for the experimental design. Additionally, the more effective competition of retinal for the binding of retinol by type II protein was not altered by the Q4F-type II mutation (Figure 4b).

Determination of Relative Affinities of Wild-Type and Mutant Binding Proteins for all-trans-Retinol. Not only were

Table 1: Competition between Wild-Type and Mutant Binding Proteins for *all-trans*-Retinol Binding

incubation reaction	% of total [^{3}H]-*all-trans*-retinol recovered			
	CRBP	F4Q-CRBP	CRBP(II)	Q4F-CRBP(II)
CRBP + 15-fold molar excess CRBP(II) ($n = 4$)	70.0 ± 6.2		30.0 ± 3.0	
F4Q-CRBP + 15-fold molar excess CRBP(II) ($n = 4$)		69.6 ± 2.6	30.4 ± 0.84	
CRBP + 10-fold molar excess CRBP(II) ($n = 10$)	67.6 ± 3.5		32.4 ± 4.1	
CRBP + 10-fold molar excess Q4F-CRBP(II) ($n = 10$)	67.5 ± 4.6			32.5 ± 6.9

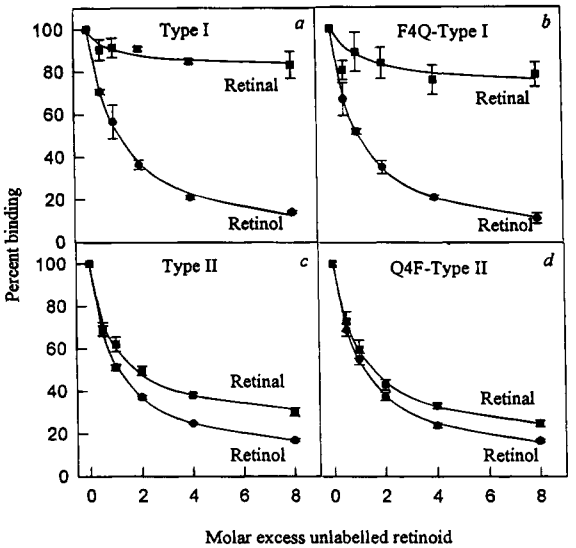

FIGURE 4: Competition between [^{3}H]-*all-trans*-retinol and unlabeled competitor (either *all-trans*-retinol or *all-trans*-retinal) for binding by (a) wild-type CRBP, (b) F4Q-CRBP, (c) wild-type CRBP(II), or (d) Q4F-CRBP(II). Experimental values are expressed as the percent of protein-associated radioactivity observed in the absence of competitor.

the mutant proteins identical to their respective native proteins in the test of specificity described above, but ligand affinities also were unchanged. Because of the modest sequence changes in the mutant/wild-type pairs, there was no convenient separation technique to allow direct mutant–native comparisons for each pair by competition for retinol binding. However, the type I and type II proteins are easily resolved by ion-exchange chromatography. We contrasted the ability of both mutants to compete with the opposite wild-type proteins for binding a limiting amount of [^{3}H]retinol and compared these results to those from wild-type-I/wild-type-II competitions. No difference in retinol affinities for either of the mutants could be detected by this procedure (Table 1).

Had the amino acid side chain at position 4 contributed to protein specificity, our mutations at this position would have been expected to alter both the affinity of the proteins for retinol and their abilities to discriminate between retinol and retinal. The fact that both mutants displayed wild-type specificities and ligand affinities indicated that the amino acid side chain at position 4, whether it be a Gln or a Phe, does not contribute to the ligand–protein interaction.

CONCLUSIONS

Burley and Petsko (1986) looked at the highly-refined structures of 33 proteins and found that side chain amino groups are preferentially positioned axially, at a distance of 3–6 Å, from the centroid of adjacent aromatic rings. Likewise, Singh and Thornton (1990) analyzed the orientations of side chain nitrogen atoms and side chain aromatic groups, within 4.6 Å of each other, in 52 high-resolution protein structures and found a similar tendency for amino groups to be located above the plane of adjacent aromatic rings. These results suggest that these side chains are involved in amino–aromatic interactions. Levitt and Perutz (1988) calculated that an amino–aromatic hydrogen bond may contribute as much as 3.3 kcal/mol of stabilizing enthalpy, which is about half the energy of a conventional hydrogen bond. However, Mitchell et al. (1994) subsequently disputed the significance of the contribution of amino–aromatic hydrogen bonds to protein stability. In their search of the protein data bank for structures which contain amino–aromatic hydrogen bonds, they imposed a strict distance criterion of 3.8 Å or less between donor and acceptor. They concluded, after examining aromatic interactions in the well-refined crystal structures of 55 proteins, that amino–aromatic hydrogen bonds in proteins are "neither common enough nor stable enough to represent a significant contribution to stability". These authors did agree that such unconventional hydrogen bonds may play a role in protein *function*, since an aromatic ring may help to orient functional groups involved in molecular recognition. This proposed functional role for amino–aromatic interactions could not be tested by their survey of refined crystal structures. Our experiments, designed to evaluate such a role for the amino–aromatic interaction in CRBP, found no contribution of the "unconventional" hydrogen bond to either ligand affinity or specificity in this protein. Our failure to note *any* contribution of the interaction to function indicates that this bond is not utilized for the orientation of Gln-108 as a hydrogen bond acceptor in CRBP. The greater affinity of CRBP for *all-trans*-retinol versus that for retinal may simply be a result of the hydrogen bond geometry of Gln-108 and the ligand polar group. This geometry is optimized for the sp^3 polar group of retinol, whereas the sp^2 polar group of retinal would be positioned such that the hydrogen bond with Gln-108 has suboptimal geometry.

The results of our direct test of the amino–aromatic hydrogen bond in protein function indicate that in this protein pair such an interaction does not play a role in ligand/molecular recognition . The importance of unconventional hydrogen bonds in other systems may also come into question.

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