The Interactions Between Cellular Retinol-Binding Protein (CRBP-I) and Retinal: A Vibrational Spectroscopic Study

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ABSTRACT: Preresonance Raman difference spectra have been obtained for all-*trans* retinal in dilute CCl_4 solution, complexed with cellular retinol-binding protein (CRBP-I) and retinol-binding protein (RBP). These spectra indicate that retinal is of a slightly more planar conformation within the binding pocket of CRBP-I than in solution or hydrophobically complexed with RBP. Compared to retinal in solution or bound to RBP, the conformation of the polyene tail of the retinal chromophore is perturbed from C8 through C11. This perturbation is probably due to the close proximity of the Lys40 in the CRBP-I binding pocket to the above-mentioned carbons. The C=O stretching vibration of bound retinal carbonyl has been found to shift from 1664 cm^{-1} solubilized in CCl_4 to 1650 and 1645 cm^{-1} in RBP and CRBP-I, respectively, and significantly broadened in both cases. The frequency shift and broadening have been attributed to hydrogen bonding. These have been compared to calibrations of frequency shift ($\Delta\nu_{C=O}$) vs. ΔH and ΔG of all-*trans* retinal complexed with a series of phenol derivatives of incremental proton-donating ability as obtained by the relationship of van't Hoff. By this relationship, the binding enthalpy of the all-*trans* retinal carbonyl moiety bound to CRBP-I and RBP is −28.1 kJ/mol (−6.7 kcal/mol) and −23.5 kJ/mol (−5.6 kcal/mol), respectively. The free energy of binding of the retinal carbonyl bound to CRBP-I and RBP has been determined to be −10.5 kJ/mol (−2.5 kcal/mol) and −7.2 kJ/mol (−1.7 kcal/mol), respectively. The hydrogen-bonded C=O moiety of retinal complexed with CRBP-I accounts for a substantial (25%) but not overriding amount of the binding energy of CRBP-I for retinal, and it also accounts for the protein's preference for binding retinol. © 1997 John Wiley & Sons, Inc. Biospect **3:** 131–142, 1997

Keywords: cellular retinol-binding protein; retinol-binding protein; vibrational spectroscopy; Raman spectroscopy; FTIR

INTRODUCTION

The importance of retinoid-binding proteins in the physiologic trafficking of vitamin A metabolites is well known. There are two distinct families of proteins that have been isolated from various tissues and capable of binding retinoids with high affinity. One family consists of highly homologous proteins. Amongst these are cellular retinol-binding proteins (CRBP-I and II), P2 myellin protein, adipocyte lipid binding protein (ALBP), and fatty acids-binding protein (FABP). The second family, which includes serum retinol-binding protein (RBP), β-lactoglobulin, and billin-binding pro-

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teins, exhibits lower sequence homology but similar folding topology. The structures of many of these proteins have been elucidated by X-ray crystallography.[1–5] Despite similarities in structure, differences in binding specificities between these families indicate differences in the motifs of their ligand binding.

Indicative of the differences between these families, two proteins, RBP and CRBP-I, provide an interesting contrast in their binding of retinal (vitamin A aldehyde). Evidence exists for low specificity of binding for RBP to various retinoid end groups.[6] This finding is well supported by a recent preresonance Raman spectroscopic study by this laboratory,[7] which indicates that retinal is bound to RBP in much the same way as retinol (vitamin A alcohol). Moreover, there was no discernible conformational change from an RBP-bound retinoid in comparison to one that is free, in a hydrophobic environment. This result is in agreement with absorbance spectra results, displaying no change in the absorption maximum of 325 nm of retinol that is RBP bound or solvated in CCl_4. Studies of the thermodynamic parameters contributing to retinol-RBP binding[8] indicate that this binding is stabilized by the entropy with little or no effect arising from binding enthalpy. It is also important to note that the X-ray studies of Cowan et al.[1] indicate that the end group of the retinoid is located at the mouth of the binding pocket.

CRBP-I offers an interesting contrast to RBP in terms of its binding affinities. CRBP-I shows a clear preference for the binding of retinol over retinal.[9,10] Cowan et al.[5] observed the chromophore to be of a more planar conformation within the binding site of CRBP-I than in solution. This observation is in agreement with the 25-nm red-shifted absorption maximum due to retinol bound to CRBP-I. The same study indicated interactions between Lys40 of the CRBP-I and the isoprene tail of the retinol. Finally, the end group of the retinoid ligand is buried deep within the binding site.

Given these differences, a comparative vibrational spectroscopic study of binding effects upon all-*trans* retinal bound to these proteins would prove informative. Preresonance Raman spectroscopy of retinal bound to RBP[7] indicates that the carbonyl of the RBP-bound retinal is exposed to a hydrogen-bonding environment. This is indicated by a 15 wavenumber red-shift and broadening of the C=O band. Several workers have demonstrated that this type of effect, first noted by Badger and Bauer,[11] may be used to quantitate the strength of hydrogen bonding as a function of the carbonyl shift.[12–14] The utility of this type of experiment to characterize enzyme function has been demonstrated by Tonge et al.[15] and Deng et al.,[16] who studied hydrogen-bonding forces involved in the complexation of enzyme and substrate and catalytic in acyl-serine proteases.

The thrust of the work presented in this study is twofold: first, to use preresonance difference Raman spectroscopy to ascertain conformational effects upon all-*trans* retinal bound to CRBP-I in comparison to those of all-*trans* retinal bound to RBP. Second, this study uses Fourier transform infrared (FTIR) spectroscopy to correlate the hydrogen-bonding enthalpy ($-\Delta H$) to the decrease in $\nu_{C=O}$ for retinal with a series of phenol derivatives of incremental proton-donating ability. This correlation is then applied to preresonance Raman spectra of retinal in the binding site of RBP and CRBP-I to facilitate a quantitation of the degree of hydrogen bonding to the aldehyde of the retinoid in both protein–ligand complexes. A quantitative comparison of this effect in both systems would help to characterize the nature and extent of hydrogen-binding forces in the binding sites of these retinal-binding proteins and to the aldehyde of the bound retinal.

MATERIALS AND METHODS

All-*trans* retinal, carbon tetrachloride, ethanol, phenol, 3,4-dimethylphenol, 3,5-dichlorophenol, 4-bromophenol, and 4-nitrophenol (Aldrich Chemical Co., Milwaukee, WI) were of the highest commercial grade and used without further purification. CRBP-I in the pMON expression vector was the generous gift of M. Levin (Department of Medicine, Washington University School of Medicine, St. Louis, Missouri), and was expressed and purified as described earlier.[17]

Protein Complexes

Concentrations of apo-CRBP-I and CRBP-I were determined from their extinction coefficients.[18,19] CRBP-I–retinal complexes were formed by mixing substoichiometric amounts of retinal (from a concentrated ethanolic stock) with purified CRBP-I at a retinal : protein molar ratio of 0.9 : 1. The protein was then concentrated and ethanol removed by three consecutive washings on a Centricon 10 Centrifugal concentrator (Beverly, MA) to a final pro-

tein concentration of 0.4 mM in a 20-mM sodium phosphate, pH 7.4. All procedures were carried out at 4°C in dim red light.

Spectroscopy

Raman spectra were obtained with a Triplemate spectrometer (Spex Industries, Metuchen, NJ) equipped with a charge-coupled device (CCD) detector (LN/CCD-1152UV) cooled to −100°C and interfaced with an ST-135 CCD controller. Samples were excited by the 752-nm line from an Innova 400 krypton laser (Coherent, Palo Alto, CA) at 60 mW. Using the 752-nm wavelength for excitation ensured no photoisomerization of the retinal, as judged from the fingerprint region of the chromophore. Residual contribution from classical (nonresonance) scattering from the protein (e.g., amide I band and aromatic residues) was digitally subtracted as described earlier.[20] Spectra were acquired and analyzed on a Mac-II computer equipped using the Igor software package (WaveMetrics, Lake Loswego, OR). Spectral lines were calibrated to established Raman lines of toluene spectra. The band assignments are accurate to within ±2 cm^{-1} and spectral resolution was 6 cm^{-1}.

Fourier transform infrared spectra were acquired on a Digilab FTS-40 spectrometer equipped with a DTGS detector. Spectra were collected at 4 cm^{-1} under the purge of clean and desiccated air, by signal averaging 64–256 scans.The spectra were apodized with a triangular function and Fourier-transformed with one level of zero filling to yield data encoded every 2 cm^{-1}. Samples were contained in a thermostated transmission cell (Harrick Scientific, Ossining, NY) equipped with KBr windows and a 0.5-mm Teflon spacer. The temperature was controlled with a circulating water bath and monitored to within ±0.1°C by a digital thermocouple (Physitemp, Inc., Clifton, NJ). Three to five independent van't Hoff plots were constructed for each donor–retinal complex studied. Spectra were analyzed using software generously supplied by D. Moffat (National Research Council, Canada). Residual water vapor contribution was corrected for by subtracting water vapor spectra recorded under identical conditions. Quantitation of free OH for the phenol derivatives was accomplished by integrating the spectral area between 3540 and 3635 cm^{-1} for a donor of known concentration, to establish molar absorbtivities for the molecules in question. To assign band positions for free and hydrogen-bound retinal carbonyls in spectra where bands from hydrogen-bonded retinal carbonyls significantly overlap the C═O absorption of free retinal, a spectrum of free retinal recorded under identical conditions of aperture and resolution was subtracted from that of the donor–acceptor complex.

Hydrogen-Bonding Studies

Measurements of hydrogen-bonding enthalpies (ΔH) between all-*trans* retinal as acceptor and a series of phenol derivatives (phenol, 3,5-dichlorophenol, 3,4-dimethylphenol, 4-bromophenol, and 4-nitrophenol) as proton donors were performed using CCl_4 as solvent. Concentration relationships were obtained using FTIR absorption spectroscopy to establish a ratio between the integrated areas of the OH-stretching vibrations in the region of 3600 cm^{-1} for the retinal–donor complexes to those of free donor of known concentration by the above-mentioned IR spectroscopic measurements. To establish their equilibrium constants (K_{eq}), the following equation was applied:

$$K_{eq} = \frac{a - x}{x(b - a + x)} \tag{1}$$

where x is the concentration in moles per liter of free phenol as determined above, and a and b are formal concentrations in moles per liter of donor and receptor, respectively. These measurements were repeated at five different temperatures between 275 and 320 K. Concentrations of retinal were varied from 0 to 50 mM, while donor concentrations were varied only in the single millimolar range, to prevent self-association. Measurements of ΔH were then obtained plotting K_{eq} vs. $1/T$ to construct van't Hoff plots from which both ΔH and ΔS were extracted.

RESULTS

The preresonance Raman spectra of all-*trans* retinal in dilute CCl_4 solution and when bound to CRBP-I are shown in Figures 1(a) and 1(b), respectively. The experimental condition (protein-bound chromophore λ_{max} = 405 nm; λ_{laser} = 752.4 nm) were chosen such as to minimize photo-alterations in the chromophore, while still retaining significant selective enhancement of its vibrational modes due to resonance effect. Residual scattering contribution from the protein moiety

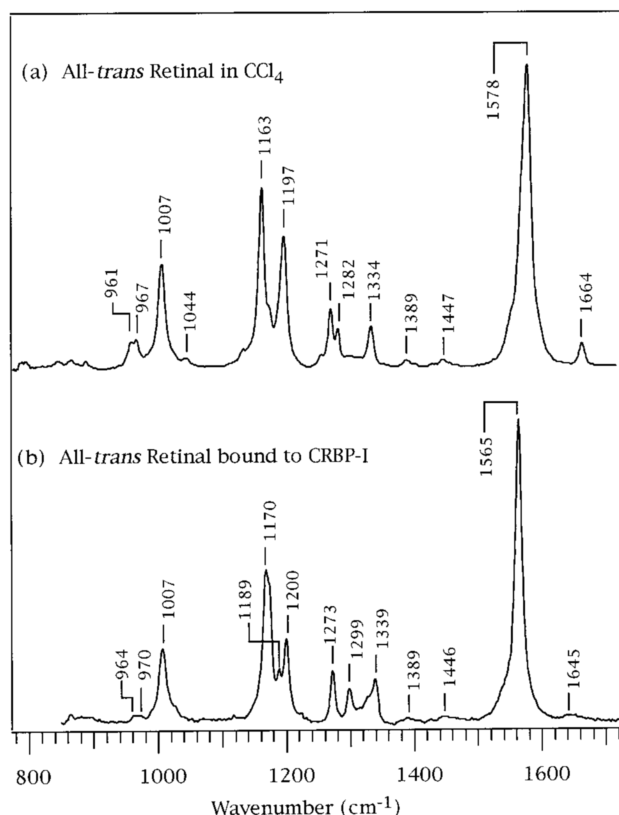


Figure 1. Preresonance Raman spectra of all-*trans* retinal in CCl_4 (a) and when bound to CRBP-I (b). All spectra were taken at 21°C with 60-mW excitation of 752-nm Kr^+ laser line. CCl_4 and apo-protein spectra were measured under identical conditions and subtracted as described previously.

(e.g., in the aromatic and amide I regions) are removed from the spectra using a subtraction procedure described earlier.[7] Rimai et al.[21–27] showed the sensitivity of the Raman spectrum of vitamin A derivatives to the isomeric conformation of its polyene chain and the disposition of the terminal functionality. A detailed vibrational analysis of the isomers of vitamin A aldehyde was performed using normal coordinate analysis as well as site-specific isotope labeling to establish assignment of the observed Raman bands.[28] These bands have traditionally been grouped into several regions according to the nature of their normal modes, and we follow this convention. Table I lists the Raman bands which change in frequency or intensity upon binding to CRBP-I, relative to their solution spectra.

Hydrogen Out-of-Plane Region

Vibrational bands in the 800–1000-cm^{-1} region of the retinal Raman spectrum are grouped into the so-called hydrogen out-of-plane (HOOP) region. These bands arise from out-of-plane bending vibrations of the *trans*-ethylenic hydrogens in the retinal polyene chain.[29] Both the number of Raman bands observed in this region and their intensity are a sensitive measure of the degree of planarity within the chromophore. Specifically, the intensities observed at 961 and 967 cm^{-1} mostly originate from HOOP motions localized *trans* to the C7=C8 and C11=C12 double bonds, respectively. As is seen in Figure 1, binding of all-*trans* retinal to CRBP-I is accompanied by decreased intensity and resolution of these two vibrational modes. Both of these are indicative of increased planarity of the chromophore, i.e., a lower torsional angle between the polyene chain and the β-ionone ring moieties of the molecule.[30] Similar conclusions were reached from earlier absorption[31] and crystallographic measurements.[5] Importantly, the same decreased intensity is observed upon the binding of retinal (vitamin A alcohol) to CRBP-I (data not shown), suggesting that both ligands are anchored in the binding pocket with similar torsional angles.

Fingerprint Region

The second vibrational region of interest in the spectra shown in Figure 1 is in the 1000- and 1450-cm^{-1} frequency range, and is known as the fingerprint region. Vibrations in this frequency region originate primarily from polyene C—C stretching motions and C—H in-plane bending motions, and therefore serve as a sensitive spectroscopic marker for the isomeric conformation of the polyene chain. For example, the spectroscopic signatures of all-*trans,* 9-*cis,* 11-*cis,* and 13-*cis* conformations were shown to differ drastically in

Table I. Vibrational Modes of All-*trans* Retinal Which Are Affected by Binding to CRBP-I

Assigned Vibration	ν_{CCl_4} (cm^{-1})	$\nu_{CRBP\text{-}I}$ (cm^{-1})
HOOP	961; 967	963; 970 weak
C10–C11 stretch	1163	1170
C8–C9 stretch	1197	1189 + 1200
7H–8H rock	1282	1299
14H rock	1334	1339
C=C	1577	1565
C=O	1664	1645

Frequencies are taken from Figure 1; their assignment is based on previous labeling and theoretical studies.[28]

this region,[21–27] and later used as a diagnostic tool for elucidation of the isomeric content of various retinal-based visual pigments.[32–36] Comparison of our spectra (e.g., Fig. 1) with those obtained using the rapid-flow techniques[37] indicates that our experimental conditions do not induce any photoisomerization of the chromophore, even when studying the most photo-labile isomer, 11-*cis* retinal (not shown). Comparison of the fingerprint region in Figure 1(a,b) shows that binding to CRBP-I is accompanied by changes in several fingerprint bands (Table I), suggesting electronic and possible geometric perturbations of the polyene chain upon binding. This situation is different from the binding of all-*trans* retinal to RBP, where no changes in the fingerprint region are observed.[7]

Traditional interpretation of these changes often applies specific isotope labeling at different positions of the chromophore. From these studies, the 1163-cm^{-1} band of all-*trans* retinal found in Figure 1(a) has been assigned to C10—C11 stretch, the 1197-cm^{-1} band to C8—C9 stretch with some C9—CH_3 stretch, the 1334-cm^{-1} band to 14H rocking, and the 1282-cm^{-1} band to 7H–8H rocking.[28] We have drawn some conclusions and speculations from these spectra based on the previous labeling studies and the available three-dimensional structure of the protein.[1,4] A few polar amino acid residues were shown by X-ray diffraction studies to reside near the bound ligand (<3.6 Å), as well as two bound water molecules. Of particular relevance to the observed spectral changes in the fingerprint region are the proximities of Lys40 and a bound water molecule, which are within 3.6 Å from the polyene chain.[4] Thus, the shift of the 1163-cm^{-1} Raman band to higher frequencies can be attributed to interaction of the positively charged Lys40 with the C10—C11 region of the polyene chain, while the splitting of the 1197-cm^{-1} band can be attributed to decoupling of the C9—CH_3 stretch from the C8—C9 one, possibly due to the proximity of Q38 as well as the bound water molecule (H_2O-43). Close approach of Lys40 and Q38 may also be responsible for the perturbation and blue-shift of 14H and 7H–8H rocking modes, respectively, located at 1334 and 1282 cm^{-1} in the model spectrum.

Ethylenic Region

C=C Vibrations

The most intense band in the Raman spectrum of virtually all retinoids is the C=C stretching mode found at 1577 cm^{-1} for all-*trans* retinal in CCl_4 [Fig. 1(a)]. Upon binding to CRBP-I, this band is shifted to 1565 cm^{-1} [Fig. 1(b) and Table I]. The intensity of this band arises mainly from double-bond stretching (i.e., C7=C8, C9=C10, C11=C12, C13=C14) combined with contributions from the various CCH in-plane rocking vibrations.[29] The strong resonance enhancement of this intensity stems from the delocalized nature of the conjugated double-bond system across the polyene chain. This delocalization is also the origin of the strong $\pi-\pi^*$ transitions within the system, which can be visualized in the strong electronic absorption of the molecule. Indeed, a linear correlation was shown to exist between the frequency of the ethylenic-stretching vibration ($\nu_{C=C}$) and the maximal wavelength of absorption (λ_{max}) in essentially all retinoids examined.[38] The observed shift of the absorption maximum to higher wavelength (405 nm) and of the ethylenic-stretching mode to lower frequency follows this trend qualitatively and quantitatively (data not shown). The shift is best explained by the interaction of the polyene chain π system with the positively charge of Lys40 and the polar water molecule H_2O-43. It can be noted that this interaction does not decouple any of the double bonds from the conjugated system. Interestingly, this is true only to binding of CRBP-I to retinal; when the protein is complexed with retinol, the absorption spectrum (as well as the fluorescence excitation spectrum) of the complex is split into three distinguished bands.[10,39] The preresonance Raman spectrum of retinol-bound CRBP-I (data not shown) shows a main ethylenic vibration at 1582 cm^{-1} with a defined weaker band at 1625 cm^{-1}. This could be attributed to the different degrees of conjugation between the two chromophores. In retinal, the carbonyl C=O is joined to the π system of the polyene chain. In retinol, where the polar head group is C—OH, the extent of conjugation is lower, and it is therefore easier to decouple individual C=C bonds. Thus, the observed splitting in the electronic and vibrational spectra possibly reflects the extension of the conjugated system into the β-ionone ring to include the C5=C6 and C7=C8 double bonds (calculated to scatter at 1611 and 1600 cm^{-1}, respectively)[28] into the resonating π system; however, their stretching vibration is decoupled from the rest of the π system, which is shifted by the interaction with Lys40.

C=O Vibration

In general, C=O-stretching vibrations are in the 1600–1900-cm^{-1} region of the vibrational spec-

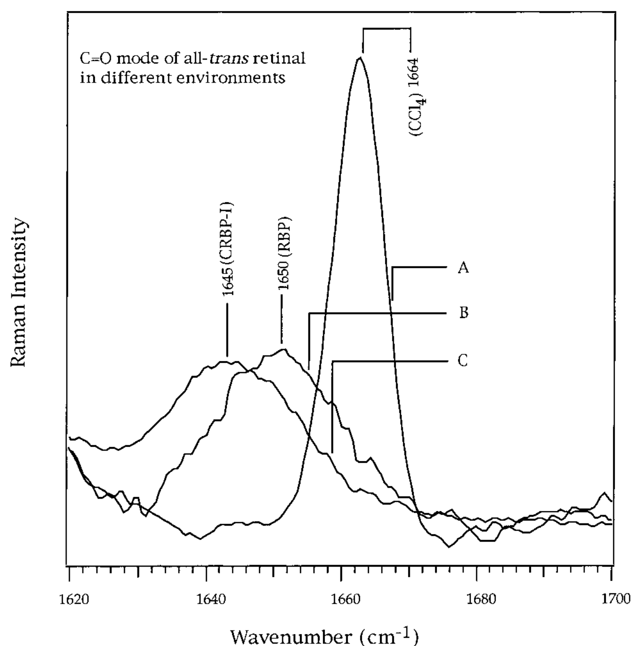
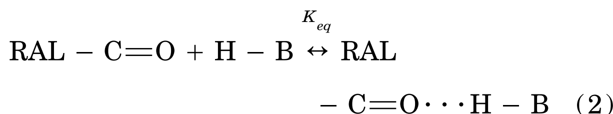


Figure 2. Preresonance Raman spectra of the carbonyl (C=O)-stretching region of all-*trans* retinal in CCl_4 [0.12 m*M* (A)] and when bound to RBP and CRBP-I [0.2 m*M* and 0.4 m*M* (B and C, respectively)]. Conditions are as in Figure 1.

trum. Conjugated aldehydes, which are capable of some degree of ketoenol equilibria due to intramolecular hydrogen bonding, tend to possess less double-bond character and therefore be in a lower frequency region, typically 1600–1700 cm^{-1}.[40] In the absence of proteic molecules (i.e., in a dilute CCl_4 solution), the C=O stretching frequency of retinal is seen at 1664 cm^{-1} [Fig. 1(a)]. Interactions with proton donors further polarize the C=O bond and decrease its bond order, hence lowering its vibrational frequency. Figure 2 shows the preresonance Raman spectrum of all-*trans* retinal in the C=O-stretching region (1620–1700 cm^{-1}) when dissolved in CCl_4 (spectrum A) and when bound to the two retinal-binding proteins, RBP and CRBP-I (spectra B and C, respectively). Comparison of the protein-bound to the "free" retinal spectra reveals two major differences: First, the maximum scattering intensity is shifted to lower frequencies (1664 cm^{-1} in CCl_4; 1650 and 1645 cm^{-1} when bound to RBP and CRBP-I, respectively). Second, the C=O-stretching vibration broadens considerably upon binding with the proteins (FWHM = 12 cm^{-1} in CCl_4, 24 cm^{-1} and 31 cm^{-1} when bound to RBP and CRBP-I, respectively, without correction of the 6-cm^{-1} spectrometer bandpass). Both of these effects were shown in model ketones to be an outcome of hydrogen bonding of the carbonyl to the proton donors.[13] To ascertain and quantitate this interaction, we undertook a systematic study of retinal hydrogen-bonding interaction with a variety of known hydrogen-bonding donors. Since the interaction involves a significant change in the dipole moment associated with the carbonyl, for accurate quantitative measurements we have used infrared absorption spectroscopy, which allows accurate concentration determination using known extinction coefficients.

The interaction can be described and quantitated as the following equilibrium system:



$$\mathrm{RAL-C{=}O + H-B} \overset{K_{eq}}{\leftrightarrow} \mathrm{RAL-C{=}O\cdots H-B} \quad (2)$$

Figure 3(a) displays the infrared absorption spectrum of the C=O-stretching region of all-*trans* retinal in CCl_4 in the presence of increasing concentrations of the hydrogen-bonding donor 4-nitrophenol. As previously seen in Raman[13] and infrared[14] studies of other model compounds, increasing proton donor concentrations results in notable red-shift of the main absorption band concomitant with significant broadening. Using known extinction coefficients for the free phenol O—H stretching region (~ 3650 cm^{-1}; see Materials and Methods), it is possible to quantitate the actual concentration of each species, thus allowing the treatment of the interaction as an equilibrium titration, as shown in Figure 3(b). It is assumed that this transition involves only two species: a free, nonbonded carbonyl absorbing at 1664 cm^{-1} and a hydrogen-bonded carbonyl absorbing at 1645 cm^{-1}. This assumption is strengthened by the fact that the titration is fitted by a single binding constant [solid line in Fig. 3(b)] and by the observation of a single isosbestic point for the spectral transition. Thus, under the experimental conditions used, there seems to be no contribution from side reactions which would complicate the analysis, such as self-association.[41] The apparent association constant extracted from this fit is shown in Table II for complexes between all-*trans* retinal and donors of varying acidity (phenol, 4-nitrophenol, 3,5-dichlorophenol, 3,4-dimethylphenol, and 4-bromophenol). Also listed in Table II are the spectral shifts observed upon interaction with the different donors and the reac-

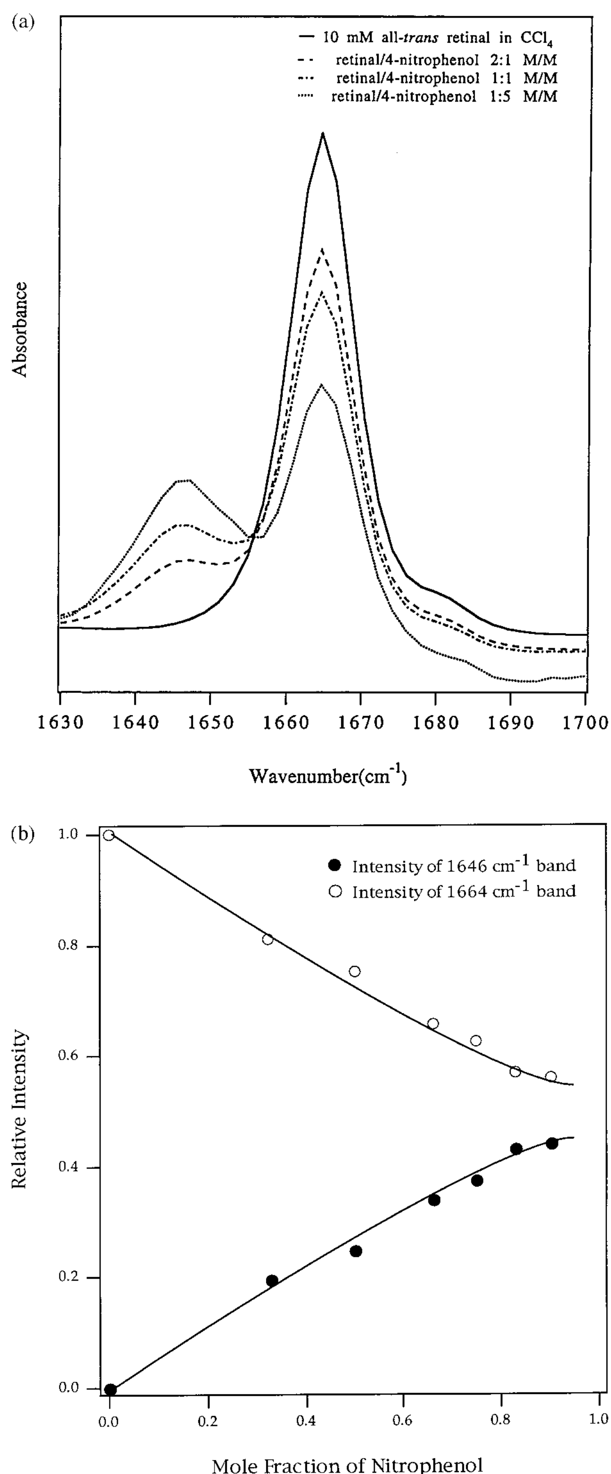


Figure 3. (a) Infrared absorption spectra of the C=O-stretching region of all-*trans* retinal in the presence of increasing concentrations of the hydrogen bond donor 4-nitrophenol. Spectra of 3–7-mM all-*trans* retinal were measured as described in Materials and Methods, after allowing the indicated donor concentrations to equilibrate for 30 min at room temperature. (b) The spectral data were converted into concentrations of the H-bonded and nonbonded retinal species as described in Materials and Methods, and plotted here as a function of 4-nitrophenol concentration. The solid line represents a fit of the data points to a single association constant (Eq. 2).

tion enthalpy as measured from the temperature dependence of the dissociation constant (van't Hoff's equation; see Materials and Methods). Further support for the notion that the observed effect is solely a result of hydrogen bonding is seen in Figure 4, from the linear dependence of the magnitude of the induced shift in the C=O-stretching frequency and the acidity (pK_a) of the hydrogen-bonding donor. When the enthalpy for hydrogen bond formation is plotted versus the magnitude of induced frequency shift ($\Delta\nu_{C=O}$), the linear correlation shown in Figure 5 is obtained. The slope of this correlation (1.13 kJ/cm^{-1}) can be used as a calibration model, from which we can obtain the hydrogen-bonding enthalpy given the magnitude of measured spectral shift.

Using this calibration, we find the interaction enthalpies of the carbonyl hydrogen bonding for all-*trans* retinal bound to RBP and CRBP-I [as shown in Fig. 2(B,C)] to be −23.5 and −28.1 kJ/mol, respectively. By a similar calibration of $\Delta\nu_{C=O}$, the free energy of association (ΔG_{298}) were obtained for the hydrogen bonding of retinal's carbonyl to RBP and CRBP-I was found to be −7.2 and −10.5 kJ/mol. Comparing this value to the overall ΔG (as derived from published association constants) of association between all-*trans* retinal and RBP (−36.7 kJ/mol) and CRBP-I (−41.6 kJ/mol) yields the fraction of binding energy that is associated with the interaction between retinal's polar group and the binding pocket.

DISCUSSION

It has been established that retinoids constitute a crucial cofactor in a variety of key physiologic processes.[42] Aside from their established role as the prosthetic group of all visual photo-receptors, vitamin A–derived molecules (retinal, retinol, and retinoic acid) appear to be involved in many crucial biological reactions, including control of cell proliferation and differentiation,[43] and are used as therapeutic agents in many malignant conditions. All retinoids are poorly soluble in

Table II. Thermodynamic Properties of All-*trans* Retinal Complexed with Phenol Derivatives of Incremental Proton Donating Ability, and Their Corresponding Carbonyl (C=O) Spectral Shift.

Donor	pK_a	Temperature (K)	K_{form}	$-\nu_{c=o}$ (cm^{-1})	$-\Delta H$ (kJ/mol)	$-\Delta G_{298}$ (kJ/mol)	$-\Delta S_{298}$ (kJ mol^{-1} K^{-1})
3,4-Dimethylphenol	10.32	281	16.34	12.7	19.9	5.84	0.047
		288	13.50				
		298	10.33				
		308	7.85				
		318					
Phenol	9.95	281	32.08	13.2	21.2	7.10	0.047
		288	26.48				
		298	20.18				
		308	14.42				
		318	11.35				
4-Bromophenol	9.34	282	61.91	16.4	24.6	8.71	0.053
		288	47.42				
		298	35.44				
		308	26.76				
		318	19.25				
3,5-Dichlorophenol	8.18	282	164.16	17.7	26.3	11.3	0.051
		288	117.08				
		298	90.52				
		308	60.84				
		318	47.99				
4-Nitrophenol	7.15	282	323.05	19.0	27.1	12.8	0.049
		288	257.27				
		298	182.22				
		308	125.62				
		318	94.05				

aqueous media, a fact which raised the search for their putative receptors and binding proteins. To date, two families of binding proteins were isolated from a variety of tissues which binds retinol and/or retinal with high affinity. CRBP-I and II belong to a family of highly homologous proteins including P2 myellin protein,[44] adipocyte lipid binding protein (ALBP),[45] cellular retinoic acid-binding proteins (CRABP-I and II),[46–48] and fatty acids-binding protein (FABP).[49] The second family, including serum RBP,[1,2] β-lactoglobulin,[50,51] and billin-binding proteins,[52,53] exhibits lower sequence homology but similar folding topology i.e., a binding cavity for the ligand composed of antiparallel β-sheets which form a highly hydrophobic pocket for solvating the hydrocarbon moiety of the ligand. One major difference between the two protein families involves the specificity of ligand binding. While RBP binds with similar affinity retinal, retinol, and many other retinoid derivatives and isomers, CRBP-I is much more sensitive to minor changes in the ligand structure. Thus, CRBP-I binds only all-*trans* retinal and retinol and a few of their geometric isomers.

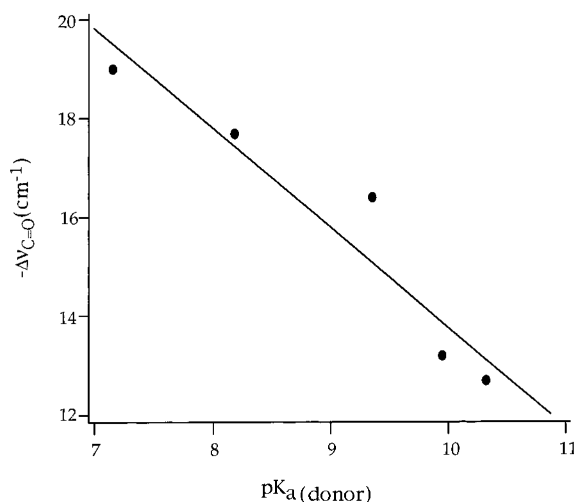


Figure 4. The induced shift C=O band frequency as a function of hydrogen bond donor acidity (pK_a).

We have previously reported a vibrational study on the interactions between all-*trans* reti-

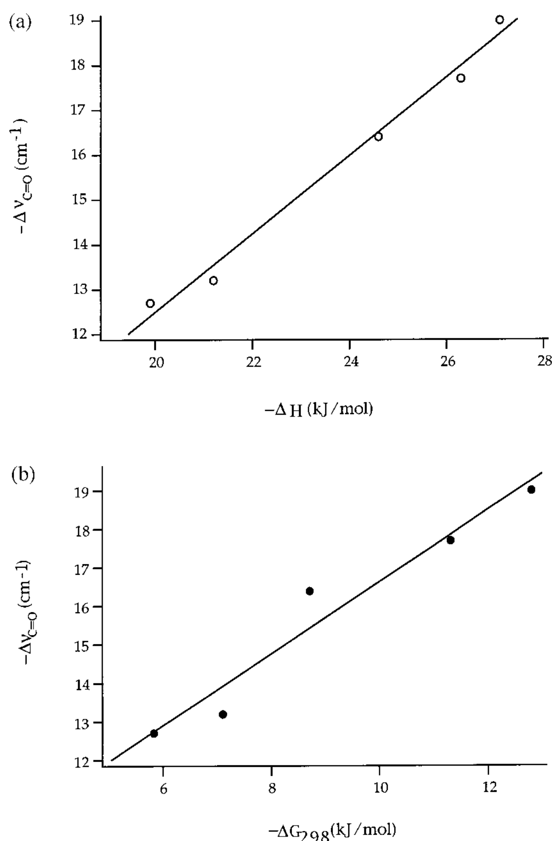


Figure 5. (a) The relationship between the frequency of the carbonyl (C═O) vibrational mode and hydrogen-bonding enthalpy ($-\Delta H$) of phenol derivatives, incremental in proton-donating ability. (b) The relationship between the frequency of the carbonyl (C═O) vibrational mode and the free energy of formation at 298 K ($-\Delta G_{298}$) for the hydrogen-bonding interaction between retinal and phenol derivatives of incremental proton-donating ability.

nal and RBP, where we demonstrated that the ligand interacts with the binding pocket solely through hydrophobic interactions.[7] The ligand-binding pocket in RBP does not contain any charged residues, and thus binding is essentially driven by an entropic contribution of water displacement within the cavity.[7,8] Some geometric changes do occur in the ligand, however, as seen by the increased intensity of HOOP region vibrational modes, which suggest increased torsional strain on the polyene chain. Supporting these data, crystallographic structural determinations of RBP[1,2] show that the chromophore β-ionone ring is positioned deep within the protein β barrel, the isoprene tail extended along the barrel axis toward the surface of the protein, and the end group exposed to water in the bulk phase. Accordingly, the RBP-bound retinal C═O band is seen to broaden and its frequency shifts to 1649 cm^{-1} from a position of 1664 cm^{-1} when the chromophore is dissolved in aproteic CCl_4. This effect has been noted previously in the spectroscopic studies of Allan and Cooper[54] and Pande et al.,[55] where incremental quantities of water was added to all-*trans* retinal in dilute acetonitrile solutions.

The nature of interaction between all-*trans* retinoid in complex with CRBP-I is one of sharp contrast with RBP, involving a number of specific interactions between protein residues and ligand. The orientation of CRBP-I bound retinol as determined by X-ray crystallographic studies[1,4] places the retinol OH functional group deep within the CRBP-I binding pocket, hydrogen bonded to the glutamine 108 residue. Lys40 of the protein makes close contact with isoprene tail, while the overall conformation of the retinoid in the binding site is planar. The preresonance Raman data presented in this study tend to augment and expand these assessments. Comparison of the HOOP mode region of the CCl_4-solubilized and CRBP-I–bound retinal spectra [800–1000 cm^{-1} of Fig. 1(a,b), respectively] indicate little or no torsional strain on the isoprene chain of the chromophore upon binding. Further agreement can be reached between X-ray and spectral data in terms of the close proximity of the Lys40 side chain and the isoprene chain of the chromophore. While specific labeling would be required to isolate Lys40 interactions with individual carbons along the polyene chain, it can be readily seen from comparison of fingerprint regions (1000–1450 cm^{-1}) in Figures 1(a,b), and the vibrational assignments of Palings et al.,[56] that positions 8–12 of the molecule are affected upon ligand binding, with the perturbation centered around the 9, 10, and 11 positions upon introduction of a positive charge from the lysine. In addition, both the X-ray data and absorption spectra agree with an interpretation of Raman data on the effect of positioning the Lys40 with the resulting effect of shifting the λ_{max} of the bound retinal absorption spectrum to 405 nm and the red-shift of the C═C-stretching band of bound retinal from 1577 to 1565 cm^{-1}, a correlation noted by Kalitani et al.[38] Examination of the carbonyl region of the bound and free retinal spectra [1620–1670 cm^{-1} in Fig. 2(A,B)] allows us to quantitatively isolate a single localized hydrogen bond interaction between protein and ligand, to evaluate its contribution to the overall binding interaction between retinal and CRBP-I. The bound retinal C═O band appearing broadened at

1645 cm^{-1} clearly is being affected by a strong hydrogen bond from within the CRBP-I binding pocket, identified in the X-ray studies of Cowan et al.[5] as Gln 108. The broadening of carbonyl band may in part be due to an increase in the number of degrees of freedom upon the retinal local binding environment due to the protein conformational changes. Interaction with the Gln108 side chain was rationalized as the differentiating factor in the preference of the protein for retinol over retinal (K_d of 10 and 50 n*M,* respectively).[9] Gln108, while accepting a hydrogen bond from the retinal OH functional group, must rotate and donate a hydrogen bond to the retinal C=O. The question then arises as to what portion of the overall binding interaction energy between ligand and protein is represented in this localized interaction, and how this quantitatively compares to other retinal-binding proteins. To answer these questions, the frequency shift of the hydrogen-bonded C=O stretching vibration as seen in the preresonance Raman spectrum of the protein–ligand complex has been assigned values of ΔH and ΔG derived from van't Hoff plots such as shown in Figures 4 and 5 and Table II. The value of −28.1 kJ/mol (−6.7 kcal/mol) is obtained for the hydrogen bond between retinal C=O and CRBP-I from this relationship. This value may be compared to −23.5 kJ/mol (−5.6 kcal/mol) for the same ligand bound to RBP, demonstrating the difference in the ΔH of hydrogen bonds between the retinal C=O and a specific amino acid or the nonspecific environment of bulk water. From the relationship of $\nu_{C=O}$ to ΔG_{298}, [Fig. 4(b)], a ΔG_{298} value of −10.5 kJ/mol (−2.5 kcal/mol) was obtained for the putative retinal C=O hydrogen to Gln108. This value may be compared to the overall ΔG of binding value of −41.6 kJ/mol (−9.9 kcal/mol) between retinal and CRBP-I, and ΔG of binding for retinol to CRBP-I of −45.6 kJ/mol (−10.9 kcal/mol), as obtained from the K_d of the complex. It is clear that the hydrogen-bonding interaction between the chromophore C=O functionality and the specific amino acid side chain accounts for only approximately 25% of the overall binding energy of the protein–ligand complex. This result is consistent with CRBP-I binding both retinal and retinol with high affinity, yet preferentially binding retinol to retinal. This type of energetic comparison would not be relevant for retinal RBP interactions owing to the difference in binding motif (see above discussion).

In summary, the preresonance Raman spectra presented in this study display a CRBP-bound retinal chromophore that is of planar conformation within the binding pocket of the protein, and whose conformation of the polyene tail is perturbed for carbons at the C8—C11 positions by the close proximity of the Lys40 residue of the protein to the chromophore. The hydrogen-bonded C=O moiety accounts for a substantial (25%) but not overriding amount of the energy of interaction, explaining both the high affinity of CRBP-I for retinal and the protein's preference for binding retinol. The bulk of the binding energy, of course, would be due to both the hydrophobic and specific interactions mentioned above. Future studies should include specific isotope labeling to further elucidate interactions between the isoprene tail of the retinal and the Lys40, as well as similar studies of other proteins within this family to ascertain if this binding motif is conserved. It is, however, significant that the differences in binding energies between RBP and CRBP-I to retinal (−36.7 and −41.6 kJ/mol, respectively) may certainly be accounted for largely by the differences in binding affinities of the retinal carbonyl alone in the respective protein–ligand interactions. The bulk of the interactions in both complexes are, of course, hydrophobic in nature.

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