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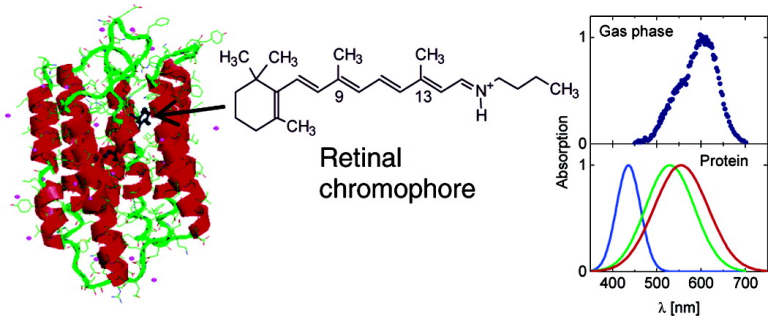

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Absorption of Schiff-Base Retinal Chromophores in Vacuo

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Abstract: The absorption spectrum of the all-*trans* retinal chromophore in the protonated Schiff-base form, that is, the biologically relevant form, has been measured in vacuo, and a maximum is found at 610 nm. The absorption of retinal proteins has hitherto been compared to that of protonated retinal in methanol, where the absorption maximum is at 440 nm. In contrast, the new gas-phase absorption data constitute a well-defined reference for spectral tuning in rhodopsins in an environment devoid of charges and dipoles. They replace the misleading comparison with absorption properties in solvents and lay the basis for reconsidering the molecular mechanisms of color tuning in the large family of retinal proteins. Indeed, our measurement directly shows that protein environments in rhodopsins are blue- rather than red shifting the absorption. The absorption of a retinal model chromophore with a neutral Schiff base is also studied. The data explain the significant blue shift that occurs when metharhodopsin I becomes deprotonated as well as the purple-to-blue transition of bacteriorhodopsin upon acidification.

Introduction

Retinal is the chromophore in many photoactive proteins, which carry out a variety of functions such as visual signaling and light-induced ion translocation. Importantly, it serves as the photosensitive moiety in rod cells, responsible for night vision, and in the photoreceptor proteins of cone cells, which provide color vision.[1−3] All vertebrate visual pigments are integral-membrane proteins containing 11-*cis*-retinal as the chromophore that is bound to opsin via a protonated Schiff-base linkage to the seventh α-helix.[4] The protonated Schiff-base form of retinal in the all-*trans* configuration is the chromophore of Bacteriorhodopsin, which is a protein in the purple membrane of *Halobacterium salinarum*.[5] Bacteriorhodopsin serves as a paradigm for transmembrane proton-pumping enzymes[6,7] and is structurally particularly well characterized.

Protein interactions cause spectral tuning of the chromophore absorption[2], yielding variations in the maximum absorption wavelength, λ_{max}. This forms the basis of color vision,[1] the understanding of which naturally is a central topic in photobiology. As absorption data for the bare retinal chromophore have not been available, comparisons are made to the absorption of protonated retinal Schiff bases in a methanol solution where the absorption reaches a maximum at about 440 nm for both *cis*- and *trans*-forms of the chromophore,[8] and the so-called "opsin shift" in proteins is defined as the shift in absorption from this value.[9] Rhodopsin in rods has its peak absorption at about 500 nm[10], whereas specific retinal−protein interactions in cone pigments tune the absorption within the region typically between 400 and 600 nm.[10] In the case of Bacteriorhodopsin,[6,7] λ_{max} is at 570 nm, that is, red shifted by approximately 130 nm as compared to λ_{max} for the chromophore in methanol.

Opsin shifts have been discussed for a long time,[11] but the precise physical origin is still a challenging open question, which is addressed in solution studies,[12] mutation studies,[13] and in high-level quantum-chemistry calculations.[14−16] A better reference for spectral tuning and for ab initio theory is the precise gas-phase absorption spectrum, that is, the energy difference between the electronic ground state (S_0) and the first excited state (S_1) without perturbations from a surrounding medium. We present

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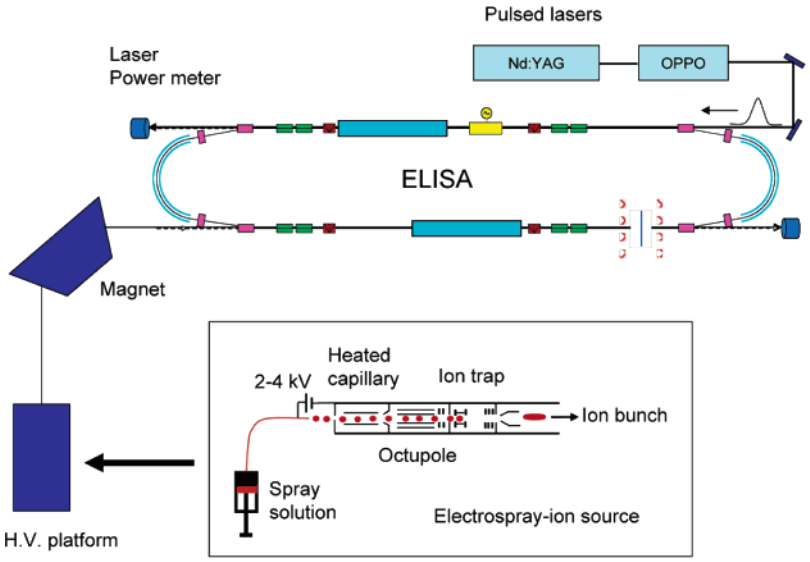
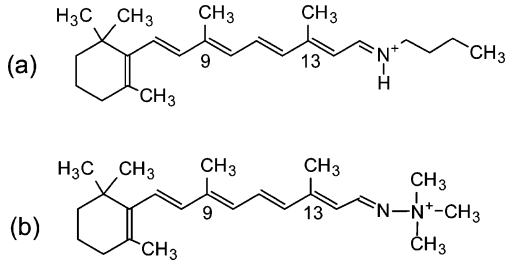


Figure 1. The ELISA storage ring with the electrospray ion source.

Scheme 1. Model Chromophores Used in the Present Work[a]



[a] (a) All-*trans* retinal with a protonated *n*-butylamine Schiff base (mass 340 amu); (b) all-*trans* retinal with a neutral Schiff base (mass 341 amu).

here an experimental determination of the gas-phase absorption spectrum of retinal in the all-*trans* protonated Schiff-base form as well as of an all-*trans* unprotonated Schiff-base form, and we compare the result with absorption in proteins and in solution.

Materials and Methods

Ion Preparation. The chromophore ions used in the present work are shown in Scheme 1. They are brought into the gas phase by an electrospray-ion source.[17] Briefly, the chromophore cations were formed by electrospray of the retinal molecules dissolved in methanol with a small amount of acetic acid. The ions were transported through a heated capillary and accumulated for 100 ms in an ion trap with a helium buffer gas, which cools them down to room temperature.

The Storage-Ring Technique. We applied the electrostatic ion storage ring in Aarhus (ELISA)[18,19] (Figure 1). After extraction and acceleration to a kinetic energy of 22 keV, the desired ions are selected by a bending magnet, and about 10 μs long bunches of typically 10^4-10^5 ions are injected into the storage ring at a repetition rate of 10 Hz. The ions circulate in the ring with a revolution time of about 75 μs until they change their mass-to-charge ratio, either by unimolecular dissociation or by collisions with the residual gas. Neutral particles formed in the first section of the ring continue on straight trajectories and hit a microchannel plate detector located at the end of this section (see Figure 1). The chromophore ions are irradiated after about 60 ms of storage. The tunable, visible laser light is provided by an optical parametric oscillator pumped by the third harmonic of an Nd:YAG laser (ScanMate Optical Power Parametric Oscillator-Lambda Physik and Infinity-Coherent, respectively, 3 ns-pulses). Tuning of the laser wavelength to an absorption band results in bond dissociation and hence in the formation of detectable neutral products. Data are accumulated in steps of 2 nm with typically 1000 injections at each wavelength.

The Signal. Figure 2 presents results obtained for the protonated Schiff-base chromophore (Scheme 1a). Shown are counts of neutral particles (normalized as explained below) as a function of excitation wavelength and time after the laser is fired. The laser excitation causes a 100−1000-fold increase in the count rate near the absorption maximum.

The signal is due to dissociation[20] caused by the ~2 eV increase in internal energy ($\lambda \approx 600$ nm). Briefly, we start with the chromophore in the singlet ground-state S_0 at room temperature where only a few of the $3N - 6$ vibrational modes are excited (N is the number of atoms in the molecule) and the average internal energy is ~0.7 eV. Photoabsorption brings the molecule into the first excited singlet state, S_1, via an intense $\pi-\pi^*$ transition,[21] and radiationless internal conversion[22] brings the molecule back to the electronic ground state with about 2.7 eV of energy distributed over the vibrational modes. This eventually leads to dissociation on the millisecond time scale (see Figure 2). Such decays have recently been modeled successfully with an Arrhenius expression for the decay rate.[20,23]

The Photoabsorption Cross Section. From a measurement of the power dependence of the yield, the photodestruction process was found to proceed through one-photon absorption. The photoabsorption cross section $\sigma(\lambda)$ is hence given as:

$$\sigma(\lambda) \propto \frac{N(\lambda) - N_0}{N_0} \Big/ \frac{\langle E \rangle}{hc/\lambda}$$

where $N(\lambda)$ is the total counts of neutral particles and N_0 is a small contribution of neutrals from residual-gas collisions. N_0 is proportional to the number of ions in the ring and may therefore be used for normalization. $\langle E \rangle$ is the average laser-pulse energy, and $\langle E \rangle/(hc/\lambda)$ is the number of photons in the laser pulse at wavelength λ.

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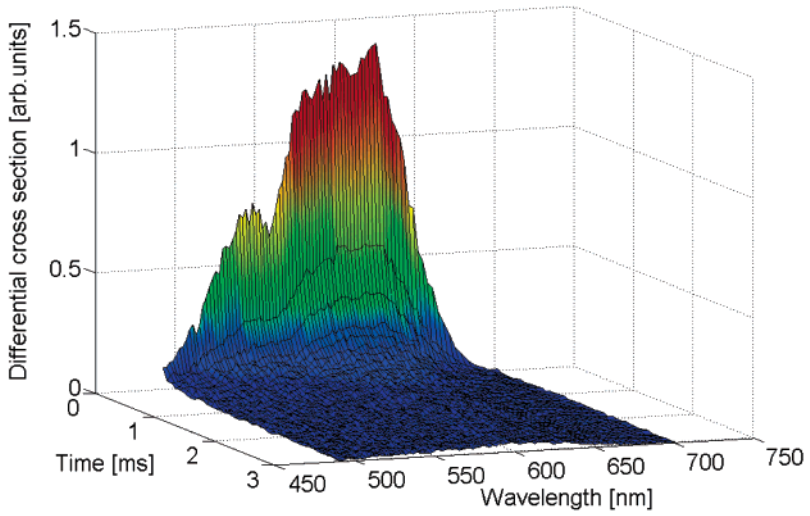

Figure 2. The differential absorption cross section as a function of wavelength and time after laser excitation (the laser is fired at time zero). Neutral particle counts are accumulated over 1000 injections at each wavelength, and the laser-pulse energy is 0.2 mJ.

Experimental Results and Discussion

In Figure 3, we show the absorption spectra of the two retinal chromophores in different environments. The absorption cross section of the isolated chromophore with a protonated *n*-butylamine Schiff base exhibits a maximum at 610 ± 2 nm (Figure 3a). The size of the 540 nm shoulder was found to vary with sample history, and the two components may hence be related to vertical transitions $S_0 \rightarrow S_1$ at different molecular structures (local minima in S_0). The measured $S_0 \rightarrow S_1$ transition wavelengths may be used as a new benchmark value for theory, which has provided values such as 501 nm (TDDFT/B3LYP),[15] 482 nm (model chromophore without the ionone ring, CASSCF),[14] and 353 nm (model chromophore without the ionone ring, CASSCF).[24] A recent calculation including the ionone ring yields 546 nm (CASPT2).[25]

The absorption spectrum in vacuo of the model chromophore of Scheme 1b is shown in Figure 3b. The Schiff base is here unprotonated/neutral, and the absorption maximum is at 487 ± 2 nm, that is, shifted by more than 120 nm to the blue relative to the gas-phase protonated Schiff-base chromophore. This has an analogy in proteins where a shift of the same magnitude is observed when metharhodopsin I becomes deprotonated.[10]

In Figure 3c, we show both the absorption of all-*trans* retinal with the *n*-butylamine Schiff base in an acidic methanol solution where the Schiff base is protonated (Scheme 1a) and the absorption of the model chromophore with a neutral Schiff base (Scheme 1b) in methanol. It is striking that the solution-phase spectrum with the protonated Schiff-base chromophore is blue shifted by 166 nm (∼6100 cm^{-1}) relative to that in a vacuum. The absorption with the unprotonated Schiff-base form (Scheme 1b) is also significantly blue shifted in the solution by 100 nm (∼5100 cm^{-1}). The solution-phase absorption properties are thus not indicative of the intrinsic electronic properties of the chromophores and are therefore not a good reference for spectral tuning of the absorption. Significant solvatochromatic blue shifts have previously been seen for chromophores of other proteins such as the Green Fluorescent Protein,[26,27] Red Fluorescent Protein,[28] and Photoactive Yellow Protein.[29] The trend seems to be that large blue shifts are observed when the electron distribution is much delocalized (highly conjugated systems) and hence sensitive to external perturbations, for example, by hydrogen bonding or counterions, which may limit delocalization of charges. In the ionic form, the retinal Schiff base and the *p*-coumaric acid are particular sensitive to the external charge distribution, for example the presence of counterions in the solvent.

The absorption spectra of three human visual pigments[30] (all with a protonated Schiff base) are shown schematically in Figure 3d. From a comparison of the gas-phase absorption maximum of the protonated Schiff-base form with the protein-absorption maxima, we conclude that the sum of all electrostatic interactions and steric constraints in the protein environment of retinal proteins, including the visual pigments, is causing significant blue shifts.

Spectral Tuning. To understand the spectral tuning, we note that the $S_0 \rightarrow S_1$ excitation of the protonated Schiff-base chromophore is a charge-transfer transition, which reduces the positive charge at the Schiff-base end.[11,14,31] There is a strong localization of positive charge at the Schiff base in S_0 and a more even distribution of positive charge in S_1. The charge of the protonated Schiff base is associated with a negative counterion in the protein, which may be important both for spectral tuning and for induced proton-transfer reactions.[4,13] A negative charge near the protonated Schiff base will stabilize the ground state more than the excited state and hence cause a blue shift in the absorption. This is fully consistent with our data because we measure the smallest excitation energy in vacuo where no negative ions are present. The effect of the positive charge in the "neutral" model chromophore (Scheme 1b) is by

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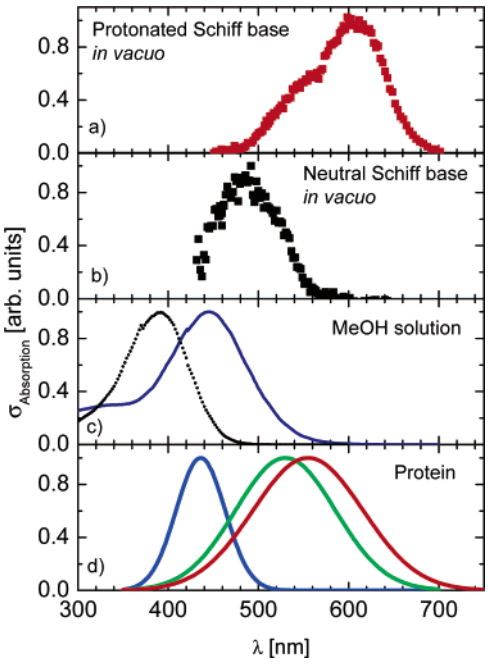

Figure 3. Absorption profiles of retinal Schiff bases in different media. (a) The absorption spectrum in vacuo of all-*trans* retinal with a protonated *n*-butylamine Schiff base (Scheme 1a) peaking at 610 ± 2 nm. (b) The absorption spectrum in vacuo of all-*trans* retinal with an unprotonated (neutral) Schiff base (Scheme 1b) peaking at 487 ± 2 nm. (c) Solid curve, absorption of all-*trans* retinal in methanol with a Schiff base (Scheme 1a) protonated with trichloroacetic acid peaking at 444 nm; dashed curve, absorption of all-*trans* retinal with an unprotonated (neutral) Schiff base (Scheme 1b) in methanol peaking at 390 ± 2 nm, both recorded by a UV−vis absorption instrument (Thermo Spectronic). (d) Absorption spectra of human visual pigments peaking at 426 nm (blue), 530 nm (green), and 555 nm (red), adapted from ref 30.

similar arguments a red shift. The absorption maximum of a "pure" neutral Schiff-base retinal chromophore in the gas phase is therefore expected to be at a wavelength shorter than 487 nm.

Our data also explain the origin of the purple-to-blue transition of bacteriorhodopsin where the 570 nm absorption maximum is shifted to 605 nm upon acidification.[5,32] When acid is added to the protein solution, the negative counteranion becomes protonated (neutralized) and the chromophore then experiences an environment that is more vacuum-like (absorption 610 nm).

As the absorption maximum of bacteriorhodopsin is only 40 nm blue shifted relative to the gas-phase absorption, the negative counterion seems to be well screened in this protein as was also concluded from high-level quantum-chemistry calculations.[33] However, distortions of the chromophore also matter. The O state of bacteriorhodopsin has an absorption maximum at 640 nm,[34] even red shifted relative to that in a vacuum. This indicates that the gas-phase chromophore, like the situation in solution,[12] is not quite planar and hence does not form the most delocalized electronic state.

Conclusions

Numerous interactions may alter the absorption profile of chromophores in solutions and proteins. Such effects include hydrogen bonding, dispersion interactions, and Coulomb interactions with charged and polar groups that change the electronic energy levels and cause conformational changes. To account for the opsin shift with its present definition, the chromophore must be modeled in three media: the solution, the gas phase, and the protein. There is considerable difference between the absorption in solutions and in the gas phase, which is not of prime interest for the understanding of the spectral tuning by proteins.[16,35] With the present gas-phase absorption data, this step can be avoided and the absorption of proteins can be directly compared to that of the bare chromophore. This should offer new perspectives in terms of pinpointing the mechanisms of color tuning in visual pigments and other retinal-containing proteins.

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Supporting Information Available: The complete list of authors in ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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