S_1 and S_2 Excited States of Gas-Phase Schiff-Base Retinal Chromophores

I. B. Nielsen, L. Lammich, and L. H. Andersen

Department of Physics and Astronomy, University of Aarhus, DK-8000 Aarhus C, Denmark

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Photoabsorption studies of 11-*cis* and all-*trans* Schiff-base retinal chromophore cations in the gas phase have been performed at the electrostatic ion storage ring in Aarhus. A broad absorption band due to the optically allowed excitation to the first electronically excited singlet state (S_1) is observed at around 600 nm. A second "dark" excited state (S_2) just below 400 nm is reported for the first time. It is located $\sim$1.2 eV above S_1 for both chromophores. The S_2 state was not visible in a solution measurement where only one highly blueshifted absorption band corresponding to the first excited state was visible. Knowledge of the position of the excited states in retinal is essential for the understanding of the fast photoisomerization in, for example, visual pigments.

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The visual pigments in the eye retina contain stacks of rhodopsin proteins which hold the retinal chromophore in the protonated Schiff-base form [1]. The absorption of visible light that strikes the eye starts an ultrafast response of the molecule, the understanding of which is a central issue in photobiology. In order to explain the very first photoresponse of the chromophore, one must know the shape and position in energy of the excited states involved. If they are well isolated, the reaction is believed to take place along the first excited singlet state (S_1), which is reached from the ground state by visible light. If, on the other hand, the next state (S_2) is close in energy, it may interact with the first excited state and, hence, play a role for the reaction path and the isomerization times.

The isomerization time of the retinal chromophore is very fast in proteins (hundreds of femtoseconds) [2] but about an order of magnitude slower in solutions such as methanol [3]. Until now, there have been no measurements of the isomerization time of the retinal chromophore molecule in vacuum, and it is thus not known whether the fast response is an intrinsic property of the chromophore or a result of its interactions with the protein. A recent *ab initio* multireference second-order perturbation calculation [4] indicates that the slower isomerization in the solution phase is related to the existence of an extended energy plateau where S_1 and S_2 essentially become degenerate. Earlier, Yamamoto *et al.* found a similar effect of a negative counterion but a rather small effect of a single water molecule near the Schiff base [5].

The presence of two close lying excited states with different electronic and structural properties has led to severe complications in solution-phase studies [6]. The order of the two excited singlet states has, for example, been debated in the past [7–10], and it seems evident that the S_1-S_2 level spacing is indeed sensitive to external perturbations, and measurements obtained in different media (solutions, proteins, and vacuum) are not necessarily directly comparable.

The excited-state energies and the influence of external perturbations are naturally relevant to photobiology. Interestingly, Anfinrud and co-workers [11] have suggested a three-state (S_0, S_1, and S_2) model to explain the photophysics in bacteriorhodopsin, whereas Olivucci and co-workers [12] are in favor of a two state (S_0 and S_1) model. We here bring an experimental determination of the S_1-S_2 energy difference for the isolated chromophore which may help to determine the role of the S_2 state. To be reliable in predicting the excited-state dynamics, theory should be able to reproduce this energy difference for the unperturbed chromophore.

It is a difficult challenge to perform gas-phase measurements because of low target densities, but they are crucial because external perturbations are avoided here. We have previously reported on the position of the S_0-S_1 transition wavelength in retinal [13] and other chromophores [14–20] and have shown that there are significant perturbations in solutions, whereas there are generally smaller perturbations when the chromophores are situated in their respective proteins. Quite recently, these findings have also been established theoretically [21,22]. This emphasizes the relevance of gas-phase studies in the field of biophysics.

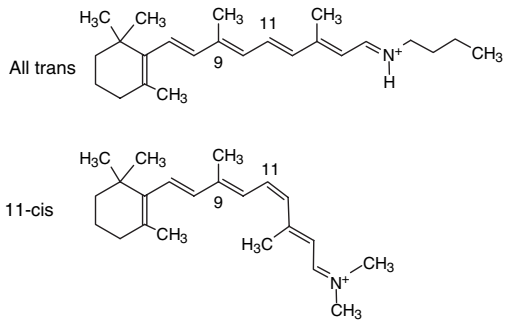

FIG. 1. Structure of the two singly charged, positive Schiff-base retinal chromophores used in the present work: all-*trans* *n*-butyl retinal ($M = 340$ amu) and 11-*cis* dimethyl retinal ($M = 312$ amu).

In the present work, we used the two retinal-model chromophores shown in Fig. 1: an all-*trans* model chromophore known from bacteriorhodopsin [23,24] and an 11-*cis* model chromophore from rhodopsin. Both have a positively charged Schiff base (*N* atom) as does the chromophore in the proteins. We thus work with charged species which makes them suitable for studies in electrostatic ion-storage devices [25], and, importantly, they are the biologically relevant forms.

The experiment was performed at the electrostatic ion storage ring in Aarhus (ELISA) [26,27] equipped with an electrospray-ion source for injection of biomolecules (see Fig. 2). The chromophore cations were formed by electrospraying a chromophore sample dissolved in methanol with a small amount of acetic acid. In the ion source, a 22-pole ion trap accumulated ions for 0.1 s before they were accelerated as an ion bunch to a kinetic energy of 22 keV. The chromophore ions were charge and mass selected by a magnet which ensured that the correct singly charged species were injected into the storage ring. The ions were irradiated 50 ms after injection by a single laser pulse in the straight section opposite the injection side. The storage before irradiation ensured that the majority of ions, which were collisionally excited during production and injection, had decayed. An optical parametric power oscillator (ScanMate, Lambda Physik) pumped with the third harmonic of an Nd:YAG laser (Infinity, Coherent) was used to create $\sim$ mJ laser pulses in the region 430–680 nm (3 ns pulse duration). In the wavelength region from 370 to 440 nm, we used an alexandrite laser (101 PAL laser, Light Age) together with a Raman scattering cell (30 ns pulse duration).

When a stored chromophore ion absorbs a photon in the wavelength region of interest here, it becomes electronically excited. As discussed in previous papers (see, for example, Ref. [28]), there is a rapid transfer of electronic energy into the vibrations by internal conversion and vibrational redistribution. Photoabsorption results in an ion with typically 2–3 eV vibrational excitation corresponding to a microcanonical temperature [29] of about 500–700 K [28]. This leads to a rather slow dissociation of the chromophore

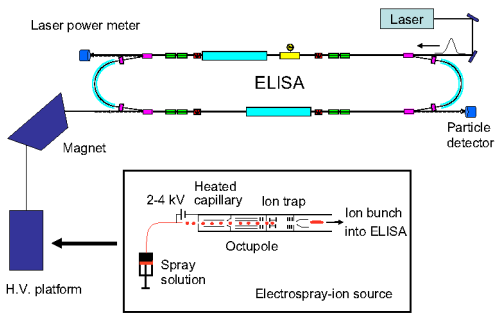

FIG. 2 (color online). The electrostatic ion storage ring ELISA equipped with an electrospray-ion source, pulsed laser, and a detector for neutral products.

ions on the millisecond time scale. The storage-ring technique is essential, since it allows us to keep the excited ions and wait for the completion of the rather slow decay (i.e., collect early as well as late decay events).

Neutral fragments formed in the straight section opposite the laser-interaction region were counted by a single-particle detector. Two examples of decay spectra (neutral-count rate as a function of time) acquired at 390 and 600 nm are shown in Fig. 3. Collisions between the chromophore ions and residual gas molecules in the ring (mainly H_2, pressure $\sim 10^{-11}$ torr) as well as unimolecular dissociation produce neutrals that account for the small background signal (see Fig. 3). It is evident that irradiation at both wavelengths results in a very significant increase in the count rate of neutrals. The decay is fastest at the short wavelength where most energy is deposited into the chromophore. At 390 nm, the absorption is ascribed to the S_0-S_2 transition, whereas at 600 nm the S_0-S_1 transition is activated (see later discussion). Going from the long to the short wavelength, the decay time changes continuously to shorter times with no discontinuity between the two electronic transitions. This shows that, for a given chromophore, the energy is dissipated in the molecule regardless of the electronic transition, and the decay is dependent only on the obtained internal temperature. Deviation from a purely exponential decay is caused by the initial energy spread of the stored molecular ensemble [28,29] and by possible trapping in metastable triplet states, the lifetime of which may be of the order of milliseconds.

The yield of neutrals was found to be proportional to the laser power at low intensity, which shows that the signal is due to a one-photon absorption process. Hence, the relative absorption cross section at a specific wavelength was obtained from the equation:

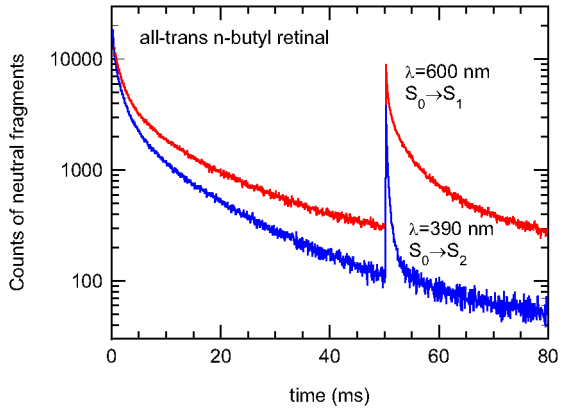

FIG. 3 (color online). Yield of neutral fragments as a function of time at 390 and 600 nm corresponding to excitation of the S_2 and S_1 state, respectively. The laser was fired 50 ms after the injection. The difference in background count rate is attributed to small changes in the ion-beam temperature from the ion source. The chromophore ion was the all-*trans* *n*-butyl Schiff-base model (see Fig. 1).

$$N_{\text{neutrals}} = CN_{\text{ions}}\sigma E/h\nu, \quad (1)$$

where N_{neutrals} is the number of neutrals corrected for the small background, N_{ions} the number of stored ions in the ion bunch, σ the photoabsorption cross section, E the laser-pulse energy, $h\nu$ the photon energy, and C a constant which depends on the experimental conditions (for example, ion-laser-beam overlap).

In Figs. 4(b) and 4(c), we show the full absorption spectrum of the two model chromophores including the bands for S_1 and S_2. As explained earlier, we used two different lasers in the S_1 and S_2 band regions. Hence, the relative strength of the absorption is somewhat uncertain because of the different laser-beam profiles. The data obtained with the two different laser systems were scaled to coincide in a wavelength region that was accessible to both lasers.

The S_1 bands show structures which are most likely due to vibrational excitation in the S_1 state. We will discuss this in detail in a future report. Here we focus on the S_2 bands. For 11-*cis* dimethyl retinal, the S_2 absorption band maximum is found at 390 nm, and the corresponding maximum for the all-*trans* *n*-butyl retinal is at 385 nm. Thus, the two chromophores have almost identical absorption maxima

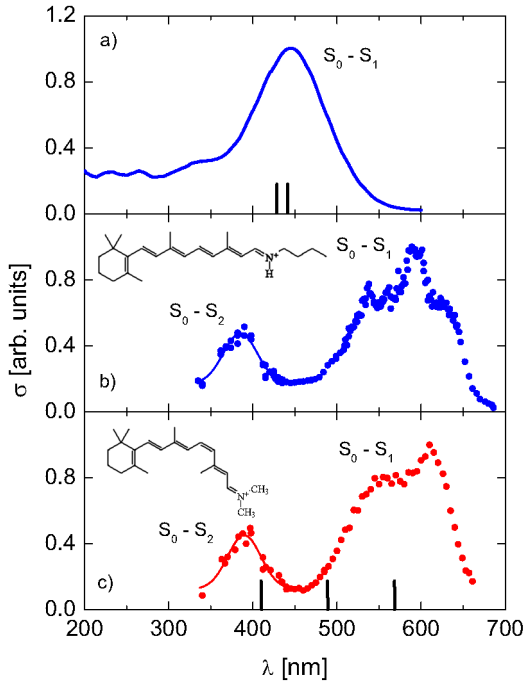

FIG. 4 (color online). Absorption cross section in arbitrary units as a function of the wavelength. (a) All-*trans* *n*-butyl protonated Shiff-base retinal in a methanol solution with acetic acid added to protonate the chromophore at the Schiff base; (b) all-*trans* *n*-butyl protonated Shiff-base retinal in the gas phase (see Fig. 1); (c) positively charged 11-*cis* dimethyl Shiff-base retinal in the gas phase (see Fig. 1). Line marks indicate the absorption maxima recorded by a previous two-photon technique in solution (a) and the protein (c) (see text).

for both the S_1 and S_2 bands. The obtained absorption wavelengths and energies are summarized in Table I. When the spectrum is recorded in a methanol solution [Fig. 4(a)], the S_1 band maximum is significantly blue-shifted by more than 150 nm, and, importantly, there is no clear sign of resolved S_1 and S_2 bands, emphasizing the need for gas-phase experiments. The two model chromophores of the present work give almost identical absorption spectra in solution.

It is interesting to compare our results with earlier measurements by Birge *et al.* on two-photon spectroscopy of protonated all-*trans* retinal in the protein bacteriorhodopsin [10] and in a CCl_4 solution [9]. We have marked the location of the two-photon absorption maxima in Fig. 4(a) (CCl_4 solution). The S_1 and S_2 states nearly coincide here, which probably explains the appearance of only one peak in the present one-photon absorption measurement. On the bottom part of Fig. 4, we mark the positions of three two-photon maxima, recorded with bacteriorhodopsin [10]. The maxima observed at 568 and 488 nm were assigned to excitation of the S_1 state (B_u-like) and the S_2 state (A_g-like), respectively. A partially resolved third peak at about 410 nm was not assigned to any particular state [10]. With the present data, it is tempting to suggest that the observed 410 nm peak in bacteriorhodopsin might be due to S_2 (slightly redshifted), and the other peaks are blue-shifted structures of the $S_0 \rightarrow S_1$ band. Further studies of the two-photon absorption of bacteriorhodopsin at short wavelength may be desirable to clarify this.

The present data may serve as a reference for theory. Recently, the S_1-S_2 energy difference was calculated to 1.19 and 1.17 eV for all-*trans* and 11-*cis* retinal-model chromophore cations, respectively [21]. This is in very good agreement with the present experiment. The calculated excitation energies $S_0 \rightarrow S_1/S_2$, on the other hand, were both overestimated by about 0.3 eV [21].

The S_1 and S_2 states are of different electronic character. At the S_0 energy minimum geometry, the S_1 state corresponds to a B_u-like (hole-pair) state, while the S_2 state corresponds to an A_g-like covalently excited dark state. The $S_0 \rightarrow S_1$ transition is associated with a charge-transfer character where the positive charge at the Schiff base is reduced upon excitation. This transition is optically allowed with a high oscillator strength ($f = 0.8$–0.9) [21].

TABLE I. The S_1 band origin is assumed to correspond to the longest wavelength peak of the S_0-S_1 absorption band.

Retinal chromophore	Transition	λ (nm)	Energy (eV)
All-*trans*	S_0-S_1	620	2.00
All-*trans*	S_0-S_2	385	3.22
All-*trans*	S_1-S_2	1017	1.22
11-*cis*	S_0-S_1	610	2.03
11-*cis*	S_0-S_2	390	3.18
11-*cis*	S_1-S_2	1079	1.15

In contrast, the $S_0 \rightarrow S_2$ transition is not associated with an appreciable charge transfer, and, as a consequence, the oscillator strength is low ($f = 0.2$) [21]. The data support the theoretical finding that the S_2 band is weaker than the S_1 band.

In conclusion, we used a storage-ring technique to measure several excited states of two biologically relevant retinal-model chromophores in the gas phase. The technique is essential for detecting the absorption, since the statistical decay into neutral fragments happens on the rather long millisecond time scale. In solutions, the excited states are either not revealed or influenced by large solvatochromic shifts. The spectroscopy of the retinal chromophore (positive Schiff-base form) is, thus, very different in the gas phase and in solutions, which suggests that there are considerable differences also in the biologically relevant excited-state dynamics. It is found that the S_1-S_2 energy difference is about 1.2 eV. The large splitting suggests that the fast photophysics of retinal containing photoactive proteins is due to the S_1 potential energy surface without much interference from S_2. The data provide a benchmark value for the energy of the S_2 electronically excited state and provide a good test of high-level theories which are being used to get to the bottom of the complicated excited-state dynamics of the retinal chromophore.

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