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Comment on “Mapping photoisomerization dynamics on a three-state model potential energy surface in bacteriorhodopsin using femtosecond stimulated Raman spectroscopy” by Z. Wang, Y. Chen, J. Jiang, X. Zhao and W. Liu, *Chem. Sci.*, 2025, **16**, 3713

I. Schapiro,^{abc} M. Olivucci,^{de} K. Heyne^f and S. Haacke  ^{*g}

The article by Wang *et al.* (*Chem. Sci.*, 2025, **16**, 3713) reports an experimental study of the photoisomerization dynamics of the all-*trans* protonated Schiff base of retinal (AT-PSBR) in bacteriorhodopsin (bR), based on femtosecond stimulated Raman spectroscopy. In the present comment, we point out a misinterpretation of a new interesting high-frequency vibrational mode, conceptual flaws, like interpreting the data in a C_{2h} symmetry framework, and most importantly the neglect of basic properties of AT-PSBR in bR, which were established over the past 30 years. The comment ends with a few suggestions on how to substantiate the new findings within a correct experimental and theoretical framework.

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Introduction

The article by Wang *et al.*¹ reports on a new study of the photoisomerization dynamics of the all-*trans* protonated Schiff base of retinal (AT-PSBR) in light-adapted bacteriorhodopsin (bR) by femtosecond stimulated Raman spectroscopy. It complements previous studies performed with the same technique,^{2,3} which were limited to a spectral range of 700–1700 cm^{-1} . However, in the following we explain in detail four fundamental points of criticism that we invite the authors to consider and comment on in their reply.

Main comment

The first aspect is technical. While previous studies were performed under low pump intensities, thus ensuring 1-photon

absorption per protein, the new study is performed with excessive intensities leading, as the authors recognize by providing the comparative Fig. S3 and S4, to slower dynamics for the decay of the J state (9.1 instead of 3.5 ps).

Unfortunately, the paper does not provide any information about the diameters of the focus spot of these beams, or does not quote the energy density (J cm^{-2}), which is the only value of importance for evaluating the percentage of proteins excited (P). Let us remind here that $P = n_{\text{ph}}\sigma$, is the product of the photon surface density of the pump beam in the focus, n_{ph} , and of the absorption cross section at the pump wavelength $\sigma(\lambda_p)$. For protein dynamics studies under relevant physiological conditions, *i.e.* under low light excitation, P should be <0.3 . This central point was unfortunately not documented in the paper.

The second and major aspect concerns the authors' central new finding: the observation of a high-energy vibronic band at 1820 cm^{-1} , which is then used to assign the electronic character of the I and J intermediates. However, such reported signature depends in a non-linear fashion on the pump energy of the excitation (actinic) pulse E_a . Indeed, an inspection of Raman intensities at 1517 cm^{-1} and at 1820 cm^{-1} presented in Fig. S5 and Fig. 2 for 50 nJ and 200 nJ excitation energy, respectively, indicates a different signal dependence of the vibrations. In a linear regime, all signals increase linearly with pump energy/intensity for all delay times. In contrast, the ratios R of the two bands at 1517 cm^{-1} and 1820 cm^{-1} at 450 fs are $R(50 \text{ nJ}) = 4.5$ and $R(200 \text{ nJ}) = 1.1$. The 1820 cm^{-1} band gains in relative intensity as compared to the one at 1517 cm^{-1} , indicating

^aDepartment of Physics, TU Dortmund, 44227 Dortmund, Germany

^bResearch Center Chemical Sciences and Sustainability, University Alliance Ruhr, 44801 Bochum, Germany

^cInstitute of Chemistry, The Hebrew University of Jerusalem, 9190401, Jerusalem, Israel

^dDepartment of Chemistry, Bowling Green State University, Bowling Green, OH 43403, USA

^eDipartimento di Biotecnologie, Chimica e Farmacia, Università di Siena, I-53100 Siena, Italy

^fDepartment of Physics, Free University Berlin, D-14195 Berlin, Germany

^gUniversity of Strasbourg, CNRS, IPCMS, 67034 Strasbourg, France. E-mail: stefan.haacke@ipcms.unistra.fr



a super-linear dependence on E_a of the former. This contradicts a linear dependence as a function of the excited state population, as it is assumed in the further analysis. We would like to refer the authors to a recent paper, where the effects of excessive excitation density (multi-photon excitation) on bR's excited state dynamics, including the increase in the lifetime of J, were reported and analysed in detail.⁴

The super-linear increase in the 1820 cm^{-1} band as a function of E_a is an observation that should have prompted the authors to investigate such non-linearity in detail. Instead, they have worked in a non-linear response regime thus (i) reporting results that are irrelevant for advancing the understanding of one-photon photo-isomerization in bR and (ii) limiting the significance of the comparison with former well-established studies on ultrafast vibrational dynamics, even if some features like the excited state Raman frequencies are well reproduced.

The third and more conceptual aspect concerns the authors' use of the above-mentioned 1820 cm^{-1} vibronic band for assigning the electronic characters of the I and J intermediates. A similar high energy vibronic signature was reported for polyenes with conjugation length $N \leq 5$, and assigned to C=C stretching (ref. 18, 20 and 21 in the paper). Based on computational results, and relying on C_{2h} symmetry, the polyene energy up-shift was attributed to vibronic coupling between the $2A_g^-$ excited state and the $1A_g^-$ ground state (ref. 19, 22 and 23 cited in the paper). However, such finding cannot be directly transferred to the all-*trans* retinal protonated Schiff base chromophore of (AT-PSBR) in bR. In fact, and in contrast with polyenes, AT-PSBR not only does not have C_{2h} symmetry but features a strongly asymmetric (polarized) π -electron density. This has the consequence that, even when adopting the common practice of retaining the C_{2h} symmetry labels, such polarized electronic structure yields a different state energy ordering that is further biased by the steric and electrostatic interactions with protein environment (Fig. 1: the ground state structure of AT-PSBR in bR is actually distorted out-of-plane). Hence, the authors' assumption that the AT-PSBR has the same electronic structure as polyene hydrocarbons is not correct and this is especially true for the further distorted I and J intermediates with non-zero dihedral angles.

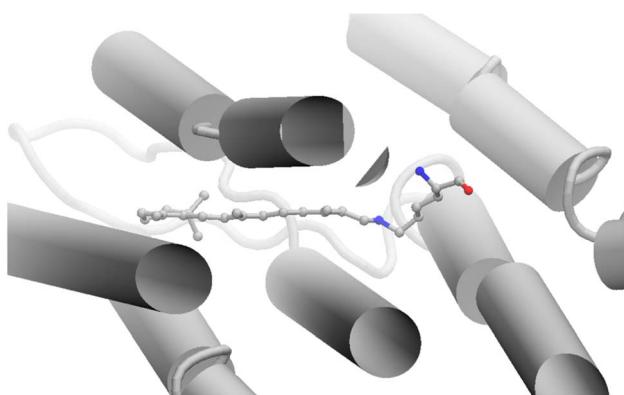


Fig. 1 Crystal structure at 1.05 \AA resolution from PDB code 7Z09.

The confusion generated by the differences in polyene and PSBRs was clarified in a review paper on simulation methods relevant for PSBR in retinal proteins.⁵ Ground and excited states of PSBR are more appropriately characterised by the degree of diabatic electronic character, namely closed shell covalent (COV) that, in terms of pseudosymmetry, could be interpreted as $1A_g$, open-shell diradical (DIR) interpreted as $2A_g$ or charge transfer (CT) that would be interpreted as a $1B_u$. This last state is now reckoned to be the low lying excited state in rhodopsins in general, a conclusion that does not apply to polyenes. The intricate mixing of the electronic characters as a function of the dihedral angle is discussed in detail in the mentioned review and should form the basis or framework for the interpretation of the FSRS data reported by Wang *et al.* Most relevantly the excited state evolution and decay occurs after propagating on a CT (*i.e.* $1B_u$) state not along a DIR ($2A_g$) state.

What is also inappropriate, is not having summarized the experimental and computational work done in the past for assigning the nature of the AT-PSBR excited state in bR and, most specifically, of the I state. In fact, when considering a valid pseudo-symmetry denomination, it has been shown to have a strongly mixed $2A_g$ (diradical)/ $1B_u$ (zwitterionic) character,^{6,7} which is abandoned when the chromophore starts to twist about the reactive double bond and enters a conical intersection formed, as mentioned above, by crossing of a $1B_u$ excited state and the $1A_g$ ground state placed half-way along the isomerization coordinate. There is instead no evidence for the crossing between the $1A_g$ and $2A_g$ states documented in polyenes, that not only would have a different energy but has a dramatically different geometrical structure not suitable for driving an efficient C=C isomerization reaction even in the gas-phase.⁵ Notice that a $2A_g/1A_g$ crossing driving the isomerization was proposed long ago but when suitable electronic structure calculations have become available it was then replaced by a $1B_u/1A_g$ picture as explained above.

The last aspects concerns the nature of the J intermediate. According to many papers on bR, J is identified as a vibrationally excited ground state population.⁸⁻¹⁴ Assigning J to a conformer with close to 90° dihedral angle (Fig. 1A in the paper) is not only at odds with this established picture, but is physically incorrect. A state with a 3 or 9 ps lifetime cannot be located at a sloped region of the potential energy surface, since the lifetime in these states is $<100\text{ fs}$ due to the presence of a conical intersection. This is also a major misinterpretation made in ref. 10, cited in the paper.

Conclusions

In summary, the observation of the vibronic band at 1820 cm^{-1} is a clear new experimental finding, but the non-linear increase in its amplitude as a function of the excitation power remains to be explained. In addition, discussing the excited states of the PSBR's in bR in terms of a putative character with C_{2h} symmetry is not only inappropriate but is in contrast with the most recent computational characterization following the diradical, zwitterionic and covalent characters associated with the $2A_g$, $1B_u$ and $1A_g$ pseudo-symmetries, respectively. We would suggest the



authors to perform a detailed study of the amplitudes of the vibronic signatures as a function of excitation power and examine the expected linear relationship. In addition, it might be helpful to perform calculations for the excited state resonance Raman spectra for bR in its I and J intermediates, in order to clarify the origin of the 1820 cm^{-1} band.

Author contributions

All authors contributed equally to this comment.

Conflicts of interest

There are no conflicts to declare.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this article.

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