Introduction and New Aspects

2

Steen Brøndsted Nielsen

Abstract

This chapter provides a brief introduction to biochromophores encountered in nature, and how their π-conjugated structures determine their excitation energies, *i.e.*, their colour. Perturbations of electronic structure by a microenvironment such as water or charge sites are discussed. These may lead to a colour change (or modulation), depending on the character of the electronic transition. As detailed results for particular chromophores are presented in subsequent chapters, future challenges and new aspects within the research field are instead considered as the author sees them.

2.1 Introduction

Chromophores are ubiquitous in nature: They account for the green colour of leaves, the red colour of blood, the orange colour of carrots, the yellow-green emission from fireflies, and the green or red emission from fluorescent proteins, just to mention a few relevant occurrences. In these examples the chromophore either acts alone, or is one constituent of a complicated network of light absorbers that play together like an orchestra where all have an important role. To qualify as a chromophore, the molecule should be able to absorb visible light. Here, however, we will extend this definition to include also molecules that absorb ultraviolet light.

This requirement for absorbing either visible or ultraviolet light implies that the relevant molecules or molecular ions are highly π-conjugated. In other words, the electronic wavefunctions are delocalised over several sp^2-hybridised atoms. A physicist will use an argument based on an electron-in-a-box with infinite walls to conclude that the larger the length of the box (the region the photoactive electron can take up), the closer is the separation between the ground-state and first-excited

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S. Brøndsted Nielsen and J.A. Wyer (eds.), *Photophysics of Ionic Biochromophores*, Physical Chemistry in Action, DOI 10.1007/978-3-642-40190-9_2,

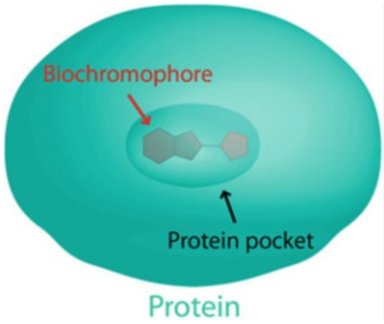

Fig. 2.1 Biochromophore buried within a protein pocket

state levels. This simple approach benefits from providing a direct estimate of the excitation energy but it suffers from not considering electron–electron interactions, and also the spacing between the energy levels increases with the quantum number instead of decreasing as they duly should. A chemist will use molecular orbital theory, and from linear combinations of atomic orbitals, the Aufbau principle and the Pauli principle reach the same conclusion regarding the intricate connection between spatial delocalisation and excitation energy based on the number of alternating double and single bonds. Here the qualitative picture is correct but a quick calculation of excitation energy is difficult, if not impossible. Examples of highly π-conjugated biochromophores include the aromatic amino acids (phenylalanine, tyrosine, and tryptophan), the nucleic acid bases (adenine, thymine, guanine, cytosine, and uracil), porphyrins and metalloporphyrins (*e.g.*, chlorophylls and heme), and several protein biochromophores responsible for example for vision, light emission, and signalling. The latter ones absorb in the visible while the former ones (aromatic amino acids and bases) absorb in the UV. Structures of some of the chromophores can be found here, in the other chapters, and in the concepts pages.

Typical ionic chromophores like the anions within the Green Fluorescent Protein (GFP) (the topic of Chap. 5 by Andersen and Bochenkova) and Photoactive Yellow Protein (PYP), the oxyluciferin anion located in the luciferase enzyme and responsible for firefly light emission, and the protonated Schiff base retinal within the rhodopsin vision protein absorb (or emit) in the visible [1]. These are buried within protein pockets or cavities (Fig. 2.1). The low energy separation between the ground and excited states is here ascribed to the fact that the ions can be represented by many resonance forms that do not involve charge separation (see Fig. 2.2 where some of them are drawn; notice the recurring phenolate moiety for the anionic photoactive molecules); all of these should be added together, properly weighted of course, to produce the resonance hybrid (linear combination of basis wave functions). In this sense these chromophores differ significantly from the nucleic acid bases and aromatic amino acids that are all overall neutral at natural pH. These ionic bichromophores, their relatives, and others isolated *in vacuo* have been exploited in great detail by both experiments and theoretical calculations [2–22], but there are still some disagreements between experiment and theory that need to be settled such as the importance of structural fluctuations potentially causing broad absorption bands.

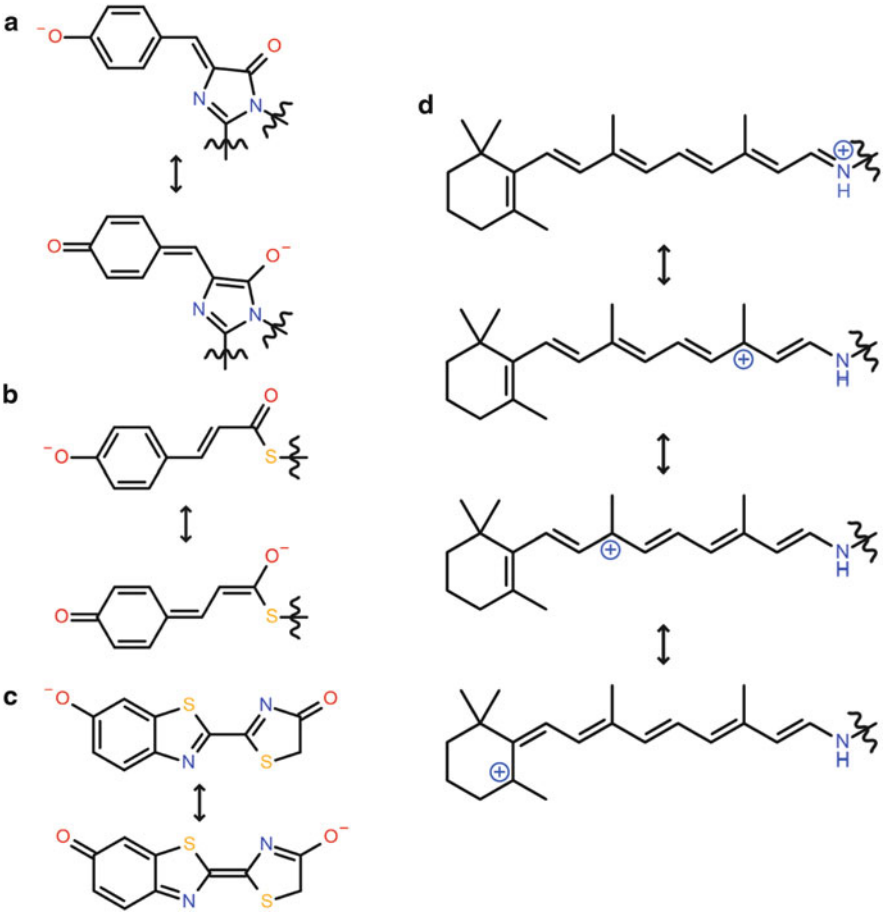

Fig. 2.2 Important resonance structures of ionic biochromophores or luminophores. (**a**) Green Fluorescent Protein: Derivative of 4-(*p*-hydroxybenzylidene)-5-imidazolinone. (**b**) Photoactive Yellow Protein (PYP): Thioester derivative of *p*-coumaric acetate. (**c**) Luciferase: Oxyluciferin anion. (**d**) Rhodopsin: Protonated Schiff base retinal. A *wavy line* indicates that the structure is joined to a polypeptide chain. The excess electron or the HOMO electron is strongly delocalised, *i.e.*, the "box length" is long

Nearby electric fields set up by an environment will perturb the electronic structure of the chromophore and influence the transition energies, dependent on the character of the electronic transition, and particularly the degree of charge transfer (CT) (Fig. 2.3). Positive or negative charges close to the chromophore can for example originate from acidic or basic amino acid residues (Figs. 2.4 and 2.5). To elucidate electric-field effects and whether an environment is innocent or not, it is useful (or even necessary) to know the intrinsic absorption spectrum of the isolated chromophore!

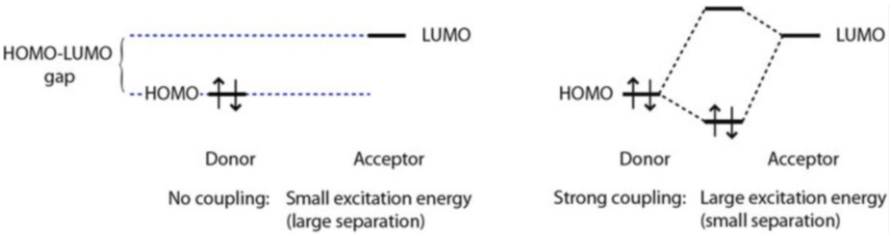

Fig. 2.3 Strong *versus* weak charge transfer. The oscillator strength increases with the degree of coupling

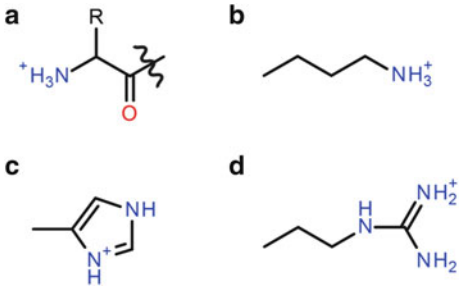

Fig. 2.4 Examples of positive charge sources: (**a**) N-terminal ammonium group, (**b**) lysine (Lys) side chain, (**c**) histidine (His) side chain, (**d**) arginine (Arg) side chain

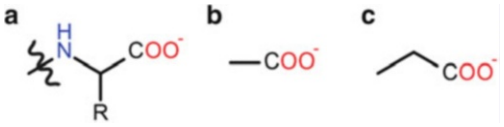

Fig. 2.5 Examples of negative charge sources: (**a**) C-terminal carboxylate group, (**b**) aspartate (Asp) side chain, (**c**) glutamate (Glu) side chain

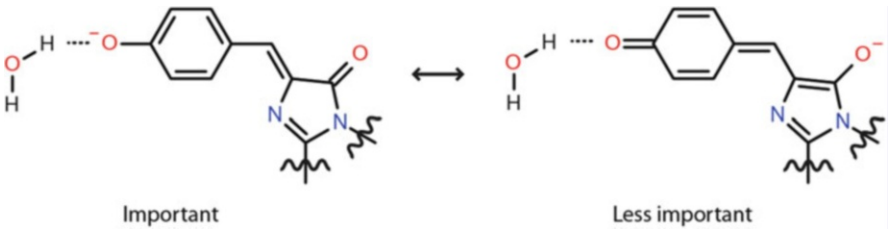

Fig. 2.6 Charge localisation by a single water molecule

Of even larger importance than nearby polar or ionic groups, could be the binding of single water molecules to charge sites (Fig. 2.6) thereby diminishing the electron delocalisation (shorter "box lengths"). However, the actual role of water molecules is not in general easily deduced as charge localisation can imply

that the electronic transition becomes more CT like (decoupling of the overlap between donor and acceptor states); CT transitions are often characterised by low transition energies and low oscillator strengths. In the extreme case of no overlap between the HOMO and LUMO orbitals (Fig. 2.3), the excitation energy is simply the difference between the ionisation energy of the donor and the electron affinity of the acceptor. Intermediate transitions are particularly difficult to account for theoretically. A picture is emerging where the situation *in vacuo* seems much closer to that of the chromophore within the protein pocket than that of the chromophore in bulk aqueous solution, at least when it comes to the chromophore's electronic structure. Still, however, both experimental and theoretical data are needed to explain each individual chromophore case.

2.2 New Aspects and Challenges

Despite the impressive amount of work that has been done in the field, some of it being presented in Chaps. 3–11, it is far from dying out, and more groups worldwide than ever are working on spectroscopy of biomolecular ions. Indeed, there are many interesting new aspects and certainly also challenges to address in the future. Some of them will be discussed in the following.

One frequent complication of the experiments is the presence of multiple isomers in the ion beam that all contribute to the electronic absorption spectrum, to the deexcitation scheme, or even to the dissociation pattern if they do not quickly interconvert. The implementation of an ion mobility device [23] right after the ion source could in many cases solve this issue as this would separate ions with different structures in time due to different drift times through the carrier buffer gas (Fig. 2.7). The combination of mass spectrometry (separation by mass) and ion mobility spectrometry (separation by geometrical structure) indeed seems a very promising and attractive direction for the future. A very recent paper by Bieske and co-workers [24] nicely demonstrates the strength of an ion mobility spectrometer combined with action spectroscopy for the study of the photoisomerisation of molecular ions. Here absorption reveals itself as a geometry change of the molecular ion resulting in a different drift time through the mobility cell! This issue of photoisomerisation is needless to say a highly relevant issue for many protein biochromophores such as the *cis-trans* isomerisation of retinal.

An action spectrum is often taken to represent the gas-phase absorption spectrum. This is in many cases a valid and reasonable assumption (see for example Chap. 3 by Wyer). However, when fluorescence is important, this may not be justified as the fluorescence quantum yield could depend on the excitation wavelength. An ambitious task would be to establish the fluorescence quantum yields for each excitation wavelength to correct for such variations. Likewise, in the photoisomerisation experiment, the isomerisation process may be wavelength dependent, and the absorption spectrum is then modulated by the photoisomerisation probability. Finally, in experiments relying on dissociation, finite time windows for measuring fragmentation can skew the action spectra to either the

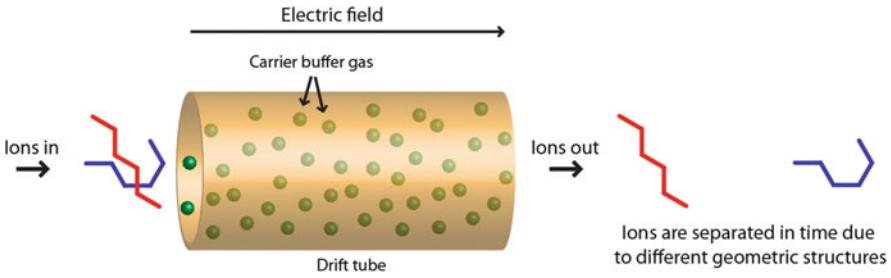

Fig. 2.7 Ion mobility spectrometry

blue or to the red. *Only when we can account for the fate of each photoexcited molecule, can we determine the actual absorption spectrum*!

Another important issue is the effect of a single solvent molecule or counter ion on the electronic structure of the biochromophore ion as mentioned above. The location of the water could seriously affect the absorption or emission properties of the chromophore/fluorophore. There is a sparse amount of data relating to this, particularly experimental data. The motivation for looking at the effect of single solvent molecules is of course that within hydrophobic protein pockets, there is limited access to water, and often there are only one or two water molecules near to the biochromophore. The advantage of experiments on ions is that such complexes can be made from say ion-molecule reactions between the bare ions and gases or even directly by electrospray ionisation, and they can then subsequently be studied by mass spectroscopic means. Furthermore, water molecules can gradually be added building up the complete solvation shell for comparison with absorption in aqueous solution. Control, selectivity and "in a stepwise manner" are the key words.

Most of the electronic spectroscopy experiments on biomolecular ions have been done at room temperature, and another important direction would therefore be to study cold ions that display much less spectral congestion and provide better benchmarks for theory. Such experiments have successfully been done with regard to vibrational spectroscopy employing 22-pole ion traps (pioneering work by Rizzo, Boyarkin and their co-workers) [25, 26] based on the original trap design by Gerlich [27]. The successful production of cold ions from the combination of electrospray ionisation with cryo-cooled Paul traps by Wang, Johnson and their co-workers [28, 29] has also paved the way for studies of for example H_2-tagged ions [29]; such complexes are easily photodissociated with visible or ultraviolet light, thereby circumpassing one of the inherent problems of action spectroscopy. Likewise, Continetti and co-workers [30] have cryogenically cooled a linear electrostatic ion beam trap for photoelectron-photofragment coincidence spectroscopy. Finally, storage rings cooled down to a few Kelvin or at liquid nitrogen temperatures also provide new interesting avenues along this direction. Such devices are built or under construction in Stockholm, Heidelberg, and Tokyo [31–33]. Spectroscopy of cold ions also provides direct information on the lifetimes of the excited states

based on the spectral width (*cf*., Heisenberg's uncertainty principle), not relying on femtosecond laser experiments. Examples on this exist and are given in Chaps. 7 and 9. The complications of environment and electronic decoupling at higher temperatures can be introduced gradually.

We believe that future work benefitting from cold ion spectroscopy could go beyond the single chromophore to multiple chromophore systems where the electronic coupling between nucleobases changes the electronic properties significantly. Interchromophore coupling is likely of importance in DNA photophysics [34, 35] but is certainly so in photosynthesis where chlorophylls are linked together electronically within light-harvesting proteins [36]. The simplicity of the gas-phase models could provide extremely important results that could be used to test advanced theoretical models such as the Frenkel exciton model. Freezing out structural fluctuations would allow one to be more specific about the actual structure of the ions, and the electronic coupling may increase significantly if π-stacking interactions determine the structures of the dominant isomers.

While fluorescence spectroscopy has been done on a few fluorophore ions *in vacuo* at room temperature (work by Jockusch, Parks, Zenobi and their co-workers [37–39]), similar experiments to those described above on very cold ions are to our knowledge lacking. Indeed, cold ions may have larger fluorescence quantum yields than warm ions as they most likely internally convert slower to the electronic ground state. Again work on multiple chromophore systems would be interesting; for example it would be worth to measure the light emission from long-lived charge-transfer states that are believed to account for long deexcitation times of DNA in aqueous solution [34, 35].

In conclusion, the field of spectroscopy of biomolecular ions isolated *in vacuo* is a relatively new field, but nevertheless the contributions made have been many and significant as is clearly evident from the chapters in this volume. Most importantly, however, the field certainly holds great promise for the future.

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Photophysics of Ionic Biochromophores
Brondsted Nielsen, S.; Wyer, J.A. (Eds.)
2013, XIII, 230 p. 125 illus., 102 illus. in color.,
Hardcover
ISBN: 978-3-642-40189-3