Photochemistry in Siegen

Günther von Bünau, University of Siegen

Preface

For an emeritus, recently resigned from his duties as a co-editor of the *Advances in Photochemistry*, it is a privilege to be asked to contribute to *The Spectrum* a report on photochemistry done at the University of Siegen after it was founded in 1972. Previous research of the author, not dealt with here, had developed along two very different lines: photochemistry of heterogeneous systems, mainly solutions,\(^1\) and multiphoton ionization of metal organic compounds, mainly \(\pi\)-complexes, under the conditions of mass spectrometry.\(^2\)

In 1978 Karl Heinz Drexhage, then at Kodak (Rochester), accepted a call to the University of Siegen. As a leading authority in the field of laser dyes, Drexhage established here a very successful group preparing the dyes and investigating their properties for a wealth of applications. Among other achievements he proved the long time doubted feasibility of anti-Stokes cooling.\(^3\) He also devised a novel (patented) way of color copying. Only recent work of his group devoted to fluorescence markers is referred to in the present contribution. Two separate chapters are devoted to achievements of former students of Drexhage. One refers to Erwin Thiel and his group working on ingenious photochemical measuring techniques. The other draws attention to a high-tech spin-off company managed by Christoph Zander at the University of Siegen. ATTO-TEC Siegen\(^4\) produces probes and devices for bioanalytical assays of utmost efficiency.

After the author retired in 1995 his successor Alfred Meixner carried on photochemistry in Siegen on completely new grounds.\(^5\) In this report the wide scope of his approach to the photochemistry of single molecules can only be touched on briefly.

Dyes for Biochemical Applications (Drexhage)

The availability of dyes with high chemical stability and high quantum yields of fluorescence, particularly in the red and near infrared spectral regions, has been a challenge for the development of dye lasers for decades. In recent years additional attention was paid to these substances as fluorescent markers for medical diagnostics and biochemical investigations, such as rapid DNA sequencing.\(^6\) In 1999 the high demand for these applications lead to the foundation of ATTO-TEC Siegen, one of the leading companies in this field (vide infra). Established techniques for labeling of biological compounds are based on rhodamines, cyanines and oxazines. Recently amidopyrylium and carbopyronin dyes\(^7\) were prepared and tested as markers, Figure 1. They fluoresce in the red spectral region, sufficiently apart from the blue and green regions where biological material often exhibits fluorescence of its own. An additional advantage of long wavelength absorbing and fluorescing dyes is the availability of inexpensive and effective excitation sources, for example, laser diodes.

Both classes of compounds are related to rhodamine and cyanine dyes, but their absorption and fluorescence is shifted considerably towards longer wavelengths. While the mesomeric structures of the carbopyronins are symmetric, those of amidopyrylium dyes are not. Absorption and fluorescence bands of the latter are broader and exhibit larger Stokes shifts. As typical examples of the

*Continued on page 3*
From the Executive Director

D. C. Neckers, Executive Director, Center for Photochemical Sciences, Bowling Green State University

A year ago the United States was reeling from the effects of the September 11, 2001 attacks. We remain vigilant in America and this has a number of implications. Americans find air travel less than pleasant; foreign visitors find obtaining visas more difficult; universities now refuse research contracts because the federal government insists on restrictions and confidentiality; and our current President is beating the drum for a war with Iraq.

The business climate in America, and probably elsewhere, is similarly problematic. There’s discussion of deflation rather than inflation and salary reductions rather than raises. Layoffs have become more common even among professionals. Several of our former students are either uneasy about current jobs, or have been forced to find new employment. The boom of the communications revolution of several years ago has become one big fat bust. Suddenly all those undergraduates who were recently so interested in “computer science” have been struggling to think of another major.

In spite of all this, (I may be the odd man out), I’m exceedingly optimistic about the immediate future. Scientists particularly are really needed right now. The economies of our states, particularly those in the Midwest, are in need of new businesses and business opportunities. Technology initiatives, like Ohio’s Wright Center and the Third Frontier programs, are specifically targeted at our university research laboratories, researchers and programs. Ohio’s Third Frontier program will place several large research centers around the state where collaborative groups of scientists will attack major problems like “alternative sources of energy”, “fuel cells”, or “polymeric materials”. Photoscientists and our Center for Photochemical Sciences will be right in the middle of such initiatives.

In Ohio we are also talking seriously about the first, in the United States, statewide broadband network. When it is in place, scientists will be able to use the ultrafast spectrometers of the new Laboratory for Kinetic Spectrometry at Bowling Green from anywhere in the state. All an attendant will need do is appropriately place a sample, and the experimenter at a distance can control the spectrometer, accumulate data, and analyze the results. Small industries will be able to use our atomic force microscopes to analyze surfaces, interrogate the integrity of coatings, and understand surface oxidation and corrosion on their samples at their desks or in their offices or labs. Again, all an operator will do is place the sample and find the image. Clearly, Center for Photochemical Sciences’ photoscientists will be among several in Ohio making their techniques “operator friendly from a distance”.

At a meeting at which I spoke in Osaka last month, I asked another speaker a question about the origin of a word he used frequently in his talk. Neither he nor anyone in the audience knew the answer to my question, but within five minutes someone “attending the talk” on the Internet sent me the origins and derivations of the word.

With all of this communications power, and all of the vexing problems of health and welfare that need be solved, how can anyone be anything but extraordinarily optimistic? I am, and I hope most of you are as well.
many individual dyes investigated we consider I with a quantum yield of fluorescence of about 0.5 and a Stokes shift of the order of 45 nm, Table 1. On the other hand, the carbopyronin dye II has a symmetric structure, a high fluorescence quantum yield and a Stokes shift of only 22 nm.

The applicability of these dyes as biological markers rests on reactive substituents that can be covalently bound to nucleotides, proteins, and so forth. Efficient coupling to amino groups of biomolecules is, for example, possible with reactive esters. They are obtained by transformation of carboxyl groups with N-hydroxysuccinimide. The optical properties of both amidopyrylium and carbopyronin dyes are unaffected by various substitutions, such as the introduction of carboxyphenyl groups. With such substitution (Table 1) both classes of compounds are no longer vulnerable to nuleophilic attack by hydroxyl ions and are stable in a wide range of pH-values.

**Figure 1. Chromophores of amidopyrylium and carbopyronin dyes.**

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>R = H</th>
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<th>R = - \text{carboxyphenyl}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption maximum λ₂</td>
<td>602 nm</td>
<td>626 nm</td>
<td>597 nm</td>
</tr>
<tr>
<td>Fluorescence maximum λᵢ</td>
<td>646 nm</td>
<td>648 nm</td>
<td>642 nm</td>
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<tr>
<td>Fluorescence quantum yield η (%)</td>
<td>47</td>
<td>51</td>
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</table>

Ultrasensitive detection of dye chromophores in marked biological molecules is achieved by the single molecule detection technique. When a solution of these molecules passes through a focused laser beam, the molecules are repeatedly cycled between the ground electronic state $S₀$ and the excited state $S₁$ which results in producing a photon.
burst. While this can be sensitively detected by a number of techniques, careful elimination of scattered light from the background and of luminescence from impurities in the solvent is a prerequisite. Rayleigh scattering and reflected light can be suppressed by suitable optical filters. Raman scattering cannot, but since it is proportional to the detection volume it can be minimized by a confocal microscope set-up with a detection volume of only a few femtoliters. Signal to noise ratios of more than 100 are readily obtained. Excitation was carried out using a pulsed diode laser at 640 nm and pulses of less than 400 ps duration at a repetition frequency of 56 MHz. Time-resolved data were acquired by time-correlated single photon counting and data processing by a personal computer. Up to three mononucleotides could be separately identified on the basis of the fluorescence life times of the attached dye molecules.

**New Techniques for Measuring Intersystem Crossing Rates (Thiel)**

Modern laser techniques have been developed to investigate ultrafast photochemical reactions. Time resolutions in the femtosecond range require highly coherent radiation as of dye lasers. Coherence is also necessary for extreme spatial resolution when the laser beam is focused by a suitable lens. Thiel and coworkers have applied this idea to the study of photochemical processes in the microsecond range.9 Basically, a fast flowing solution is exposed to a focused laser beam and probed by another downstream, Figure 2. Given a flow velocity of $50 \text{ m/s}$ (= v) and a cross section of the focus of $5 \mu \text{m}$ (=$\Delta x$) a time resolution of $0.1 \mu \text{s}$ (= $\Delta x/v$) is obtained.

Fast flowing photochemical systems are well known from continuous dye lasers. A solution of the dye is pressed through a nozzle producing a stationary jet. Since the flow must be laminar, only certain solvents are suitable. Ethylene glycol has proven to yield jets having plane parallel surfaces and excellent optical properties. Linear flow velocities are, however, restricted to about $10 \text{ m/s}$ 10. As an improvement Thiel and coworkers have designed annular cuvettes that are revolved at 150 cps.11 With a cell diameter of 15 cm linear velocities amount to more than $60 \text{ m/s}$. Arbitrary solvents can be used in this arrangement.

Many important photochemical processes take place in the time range of microseconds. Examples are intersystem crossing and quenching reactions leading from emitting (often fluorescent) states to dark states (often triplets). When high quantum yields of fluorescence are essential, as in fluorescent labeling of biochemical material, intersystem crossing is an unwanted side reaction competing with the emission process. Measuring its rate is then of principal interest.

Compared with internal conversion and fluorescence transitions intersystem crossing is a slow process. Accordingly, resultant transients are present only in low concentrations and require sensitive detection. Thiel and his group have developed a sophisticated transient spectrometer of very high sensitivity.12 It is based on mechanical chopping of both the exciting and the probing laser beam and on lock-in amplification of modulated detector signals. Modulating the exciting beam intensity at a given frequency $\omega_E$ allows suppression of background light. However, fluorescence stray light is produced modulated with the same frequency. Its influence can be eliminated when the probing beam intensity is modulated at a frequency $\omega_P \neq \omega_E$ and the detected signal is recorded after lock-in amplification at the sum frequency $\omega_P + \omega_E$. Because of the stationary movement of the sample, long signal integration times provide for highly sensitive detection of transients.
Alternatively, transients were investigated by measuring fluorescence intensities of the moving sample instead of absorptions. This approach is based on the depletion of the ground state paralleling the population of transient states. A laser beam is split up into branches of equal power passing at separate points through the sample as in Figure 2. Because of ground state bleaching fluorescence intensity is lower at a focus downstream and related uniquely to the ground state concentration. By chopping the two beams at different frequencies and lock-in amplification of the resultant photo current relative changes of the fluorescence emission of the order of $10^{-5}$ can be detected.

The techniques were applied to dyes of interest as biological markers. Rates of intersystem crossing to the triplet state and from there to the ground state were measured in the presence and absence of oxygen and interpreted on the basis of a Jablonski diagram, Figure 3. Selected rate constants are reproduced in Table 2. They are in excellent agreement with previous data.

<table>
<thead>
<tr>
<th>Table 2(^{11})</th>
<th>(\text{Intersystem crossing rate constants for Rhodamine 6G, cf. Figure 3})</th>
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<tr>
<td>Emitting state (E) &amp; Dark state (D)</td>
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<tr>
<td>(k_{ED} = k_{\text{IED}} + k_{\text{qED}} [O_2])</td>
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<tr>
<td>Dark state (D) &amp; Ground state (G)</td>
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<tr>
<td>(k_{DG} = k_{\text{IDG}} + k_{\text{qDG}} [O_2])</td>
<td></td>
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<tr>
<td>unimolecular &amp; bimolecular</td>
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<tr>
<td>(k_{\text{IED}}) ((s^{-1})) &amp; (k_{\text{qED}}) ((1\text{mol}^{-1}s^{-1}))</td>
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<tr>
<td>(6.1 \times 10^5) &amp; (22 \times 10^8)</td>
<td></td>
</tr>
<tr>
<td>unimolecular &amp; bimolecular</td>
<td></td>
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<tr>
<td>(k_{\text{IDG}}) ((s^{-1})) &amp; (k_{\text{qDG}}) ((1\text{mol}^{-1}s^{-1}))</td>
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<tr>
<td>(5.9 \times 10^4) &amp; (22 \times 10^8)</td>
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Fluorescence Markers and Devices at ATTO-TEC (Zander)

ATTO-TEC was founded to produce and sell components of extreme efficiency for applications in biotechnology and medicine, such as dyes (ATTO-Labels), chip-readers for immunoassays (Immun-o-mat\(^{®}\)), a confocal fluorescence microscope (LightStation\(^{®}\)), and schemes for high speed automatic sequencing and ultra sensitive detection of viruses, bacteria, antibodies and genes. As an example, the GenePin\(^{®}\), an intelligent DNA-probe and the way it works, is described here in some detail. GenePins are oligonucleotides comprising a designed sequence that is complementary to a target sequence in an analyte biomolecule. One end of the sequence in the GenePin carries a chain of guanosine and the other of cytosine bases: G and C in Figure 4. Thereby the uncomplexed gene-pin assumes a hair-pin structure, A) in Figure 4. In this conformation, the strongly quenching guanosine bases are close to the fluorescent label attached to the cytosine end. Owing to the proximity, the fluorescence of the label is quenched efficiently. The hair-pin conformation is opened when specific coupling of the GenePin to the target takes place. Now strong fluorescence is detected. This mechanism is ideally suited for homogeneous assays. Target sequences, as in viruses or bacteria, are unambiguously recorded in concentrations between \(10^{-7}\) and \(10^{-12}\) M within a few seconds. This is more sensitive by at least three orders of magnitude in comparison with former tests.
The Spectrum

Common fluorescence spectra and lifetimes of ensembles are averages obscuring details of conformation and of interactions with the environment. In contrast, spectral properties of individual molecules exhibit fluctuations that are not well understood and subject to speculation. Usually they are ascribed to environmental influences. Spectral changes resulting unambiguously from transitions between different conformations were first observed in single molecule experiments with the substances API (= 9-amino-N-(2,6-diisopropylphenyl)-perylene-3,4-dicarboximid) and its derivative DAPI (= N,N-di-(tert-butoxycarbonyl)-API)\textsuperscript{13,14}, Figure 5.

Ensemble measurements show that absorbance and fluorescence spectra of the two compounds are very different, Figure 6. DAPI-spectra exhibit distinct vibronic structure and are shifted hypsochromically relative to API. The fluorescence quantum yield of DAPI (0.7) is much higher than that of API (0.2). When API is protonated its spectra resemble those of DAPI. These findings are consequences of the change introduced into the chromophore of API by replacing the H-atoms in its amino-group by bulky COOtBut-groups, which force the nitrogen lone pair out of the $\pi$-system, Figure 7.
The ensemble spectrum of DAPI is also obtained when large numbers of single molecule fluorescence spectra, recorded at very low concentrations, are averaged. However, single API-molecule fluorescence spectra fall into two distinct groups. One represents the unstructured API bulk spectrum of low fluorescence quantum yield while the other belongs to the DAPI fluorescence spectrum of high quantum yield. This result corresponds to the two possible types of conformers of API molecules, an off-resonance and an in-resonance conformation with respect to the $\pi$-system. On this basis the observed fluorescence dynamics of the API spectra is readily understood: when rapid sequences of single molecule spectra were recorded a gradual transition from one type of spectrum to the other was observed in both directions.

Summary

Current photochemical work in Siegen includes:

- the development of dyes of high fluorescence quantum yield in the red and near infrared spectral regions as fluorescence markers in biochemical applications; studies of interrelations between fluorescence properties and structures of dyes (Drexhage),
- novel techniques for measuring intersystem crossing rates (Thiel),
- the production of probes and schemes for high speed automatic sequencing and ultra sensitive detection of nucleotide sequences (Zander),
- laser spectroscopy with optical detection of single molecules and high spatial resolution on surfaces (Meixner).

References

4. www.atto-tec.de and info@atto-tec.de

About the Author

Günther von Bünau received his Ph.D. with Manfred Eigen at Göttingen University in 1957. He had been a postdoctoral fellow at Notre Dame University with Milton Burton (1958-60) and worked on the radiation chemistry of gases. Continuing these studies at the Max Planck Institut für Strahlenchemie in Mülheim with G. O. Schenck his interests shifted to the photochemistry of gases in the vacuum ultraviolet region. From 1973 to his retirement in 1995 he was professor of physical chemistry at Siegen University. He can be reached at buenau@chemie.uni-siegen.de.
Intramolecular Charge-Transfer Studied by Time-Resolved Vibrational Spectroscopies

David Phillips, Department of Chemistry, Imperial College

Introduction

In August 2002, the death occurred of George Porter, the co-inventor of flash photolysis, which earned for him and Ronald Norrish a share in the 1967 Nobel Prize. The technique has been of immense benefit to the photochemistry community over the past fifty years or so, and in tribute to George Porter, what will be described in this article is a development which records the vibrational spectra and kinetics of intermediates in photochemical reactions rather than the electronic spectra conventionally recorded. Electronic spectra of complex polyatomic molecules are usually broad and featureless, and thus do not convey much structural information about the intermediate. Vibrational spectra can in principle yield quality structural information, as will be demonstrated with regard to the molecule, 4,4’-dimethyl amino benzonitrile (DMABN), a prototype for molecules which exhibit emission from an intramolecular charge-transfer state in polar solvents.

There are two complementary methods for recording vibrational spectra, viz infrared spectroscopy, and Raman spectroscopy. When applied to the small concentrations of transients produced photochemically, both are relatively insensitive, the Raman case being such that in order to record spectra, resonant enhancement using a probe wavelength close to an electronic absorption band of the transient is necessary in order to see a signal, giving rise to the acronym for the technique TR3 (Time-resolved resonant Raman) spectroscopy. Since the Raman effect is a very weak scattering phenomenon, Raman signals from strongly or indeed even weakly fluorescent molecules are swamped by the fluorescence, which is usually orders of magnitude more intense. Since fluorescent molecules are of immense photochemical interest, this has proved a barrier to exploitation of the potentially very useful Raman technique. Below is described an apparatus, developed by colleagues (listed in the acknowledgements section) at the UK National “Lasers for Science Facility” at the Rutherford Appleton Laboratories near Oxford, which permits recording of infrared spectra of transients on the picosecond time-scale, and also the TR3 spectra of even highly fluorescent samples. Used in the former mode, the apparatus carries the acronym “PIRATE” (Picosecond infrared absorption of transients experiment).

The Experiment

Both modes of operation are classic “pump-and-probe” methods requiring a tunable, intense short laser pulse for excitation of the sample. In the case of the Raman experiment, a picosecond tunable visible probe pulse is needed, whereas for the ir experiment, a tunable picosecond infrared probe is required. All can be delivered from solid-state femtosecond Ti:sapphire oscillators/regenerative amplifier systems used to pump optical parametric oscillators, OPAs. The systems are shown in Figure 1, configured for the Kerr-gated TR3 experiment, and Figure 2, configured for PIRATE measurements.

The 1 KHz repetition rate femto/picosecond dual OPA system for generating two independently tunable pulses in the wavelength range 205-2800 nm is described in detail.
elsewhere,¹ but is centred around a Ti:S regenerative amplifier (Spectraphysics Spitfire/Merlin) operating at 1 KHz, and ca 800 nm, and delivering from 0.7 to 2.5 mJ per pulse. The regenerative amplifier is seeded from a modelocked Ti:S laser (Spectraphysics Tsunami). Two modes of operation are possible with this system: (1) picosecond, in which spectral filtering of the Ti:S oscillator produces 800 nm output of ca 1-2 ps FWHM pulse duration, and 15-7 cm⁻¹ bandwidth respectively, and (2) using a femtosecond stretcher/compressor to provide an output of 150 fs and ca 100 cm⁻¹ bandwidth. The 800 nm output is frequency doubled in a BBO crystal, and split to produce two OPAs, which when combined with upconversion, (including the idler tunability), give the 205-2800 nm spectra range.

For the TR³ measurements, a high performance Kerr-gate designed for the suppression of fluorescence in both time-resolved and steady state resonance Raman spectroscopy is utilised.² The gate opens for ca 4 ps, throughput in the open state is ~40%, and the extinction ratio in the closed state is 10⁵. The usable spectral range is 300-700 nm with a single set of polarisers. The effectiveness of the device is shown in Figure 3, where the Raman spectrum recorded for the highly fluorescent dye Coumarin C480 is shown without the gate, using a subtraction technique, and with the gate. Very high quality time-resolved fluorescence measurements can be made on this apparatus by “inverting” the modus operandi, that is, suppressing the pump and Raman signals and permitting the fluorescence to be transmitted by rotation of one of the polarisers. High quality time-resolved electronic absorption measurements can also be made on this system.

For the time-resolved infrared (PIRATE) experiments, the 150 fs, ca 150 cm⁻¹ FWHM mid-infrared probe and reference pulses are generated by difference frequency mixing of near ir pulses in Type I AgGaS2, and are dispersed over two 64-element linear infrared array detectors. This gives a sensitivity of delta OD ~ 10⁻⁴ to 10⁻⁵ with 1 minute acquisition time.³

The two techniques of TR³ and TRIR are truly complementary, as will be shown by reference to the specific example below. The TR³ experiment has poorer spectral resolution, but broader spectral coverage than the TRIR, which

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Figure 2. System used for “PIRATE” experiments.

Figure 3. Suppression of fluorescence in TR³ spectroscopy. Spectra of acetonitrile contaminated with Coumarin 480. (a) without gate; (b) without gate, fluorescence subtracted; (c) Kerr-gated Raman spectrum.
currently limited to the range 100-200 cm$^{-1}$ on each scan, whereas the TR$^3$ experiment can give the whole spectrum on one accumulation. TRIR also has better time-resolution, but is limited at present to the spectral region above 1000 cm$^{-1}$. However, since not all vibrational motions are both IR and Raman active, both techniques are required.

Application to Charge-Transfer in Dimethylaminobenzonitrile (DMABN) (Structure shown in Figure 5)

DMABN has been the subject of very many studies in the forty years or so since Lippert$^4$ first reported the “dual” fluorescence of the molecule in polar solvents, yet controversy still surrounds the structure of the intramolecular charge-transfer (ICT) state which is produced upon excitation of the molecule in polar solvents and there has not been general agreement between different time-resolved studies, usually on fluorescence, regarding the kinetics of conversion between initially-produced locally excited state and the ICT state, and their decays. Many mechanisms, both monomolecular$^{5-10}$ and arguments proposing specific solute-solvent interactions$^{11-15}$ have been proposed.

There are two main contenders as models for the structural changes accompanying the ICT reaction. In the “twisted” ICT model (TICT), the ICT state is formed by twisting the dimethylamino group from its planar configuration in the locally excited state (as in the ground state), into a geometry perpendicular to the plane of the benzonitrile moiety; this is assumed to be accompanied by full electron transfer from the lone-pair of the amino N-atom to the in-plane $\pi$ orbital of the benzonitrile group.$^{16}$ The 90 degree twisted conformation would lead to electronic decoupling of the amino and benzonitrile groups, and consequent loss of conjugation between the donor (amino) and acceptor (benzonitrile) groups. In the “planar” (PICT) model,$^{17}$ the geometry change is supposed as a change from a pyramidal structure of the amino group in the LE state towards a planar configuration in the ICT state, giving a quinoidal planar resonance structure in which the donor and acceptor groups are strongly coupled.

We have over the past few years attempting to resolve some of these questions by application of time-resolved vibrational spectroscopy to this and related molecules. Readers are referred to the several full papers published to date for full details of the experiments, but a summary of our major findings will be given. A key result$^{18}$ is the identification through isotopic substitution of the spectroscopic signature of the amino N-ring carbon stretching vibration at 1281 cm$^{-1}$ which is down-shifted by $\sim$ 96 cm$^{-1}$ from the

| Table I. Comparison of observed frequency changes in DMABN ICT states with ab initio CASSCF predictions on TICT and PICT models. |
|---|---|---|---|
|   | $v$ (C=N) cm$^{-1}$ | $\Delta v$ | $v$ (Ph-N) cm$^{-1}$ | $\Delta v$ |
| $S_0$ | 2352 | 1356 |
| TICT | 2242 | -110 | 1286 | -70 |
| PICT | 2316 | -36 | 1404 | 47 |
| Kerr Gate TR$^3$ |   |   |   |
| $S_0$ | 2219 | 1377 |
| ICT | 2095 | -124 | 1281 | -96 |

Figure 4. TR$^3$ spectra of the ICT state of DMABN and isotopomers. A key vibration is the weak band at 1281 cm$^{-1}$ (see text).
corresponding band in the locally excited state. See Table 1 and Figure 4.

This result clearly favours the TICT model, in which a weakening of the bond through loss of conjugation would be expected, over the PICT model, in which a strengthening of the bond would occur, with consequent upshift of the frequency, as proposed in theoretical CASSCF work. However, it must be stated that the vibrational analysis of ground-state DMABN shows that the weak mode corresponding to the 1281 cm\(^{-1}\) ICT mode is a complex vibration, which has contributions from numerous other local modes, and this complexity weakens the conclusions based upon the frequency shift of this mode. Although we remain favourably disposed towards this conclusion, others are opposed, and the proof of either TICT or PICT models remains elusive until further structural studies on these short-lived transients can be performed.

The reconciliation of disagreements in the literature concerning the kinetics of formation and decay of the ICT states in DMABN and related molecules has, however, proved to be amenable to careful TRIR studies in the CN stretching region of the IR spectrum. In Figure 5 is shown the TRIR and ground state FTIR spectrum of DMABN in acetonitrile and methanol excited at 267 nm, and for different time-delays. It is very clear from the spectra that in the protic (methanol) solvent, two IR transient bands are visible, whereas only one is apparent in the non-protic, polar acetonitrile solvent. The new band appearing in the protic solvent is a hydrogen-bonded ICT state, termed here the HICT state.

The kinetics are summarised in Figure 6, from which it can be deduced that the ICT state is populated from the LE state, and a dynamic equilibrium between the ICT and HICT states is established on a 13 ps timescale. The internal conversion de-excitation rate of the HICT state is very much larger than that of the ICT state, and is mainly responsible for the reduced quantum yield of fluorescence in methanol solution. The overall fluorescence of DMABN in methanol is thus composed of contributions from LE, ICT and HICT states, and thus requires a three-state
mechanism for the dual fluorescence of DMABN and related molecules in protic solvents. Such a mechanism has not been proposed before, but can be shown to be compatible with results here and in the literature. The spectroscopic identification of this new hydrogen-bonded ICT state is a graphic illustration of the power of the high-resolution, high-sensitivity TRIR technique, which, when used in conjunction with the complementary TR3 method, should be capable of solving many other photochemical and photophysical problems.

References

Acknowledgements
The work described here has all been carried out by my colleagues at Rutherford Appleton Laboratories (Tony Parker, Pavel Matousek, and Mike Towrie), The University of Oxford (Bill Toner), the University of Nottingham (Mike George and David Grills), and Imperial College London (Wai-Ming Kwok and Chensheng Ma), under the terms of research grants from the Engineering and Physical Sciences Research Council, UK.

About the Author
David Phillips was awarded his Ph.D. in physical chemistry in 1964 at the University of Birmingham, UK. He traveled on a Fulbright Fellowship to the University of Texas at Austin to carry out a postdoctoral fellowship with W. Albert Noyes Jr. (1964-66), then was Royal Society/Academy of Sciences of USSR Exchange Fellow in the Institute of Chemical Physics, Moscow, USSR during 1966-67. He was on the Chemistry staff of the University of Southampton, UK from 1967-79, when he then became Wolfson Professor of Natural Philosophy and Deputy Director of the Royal Institution of Great Britain, London, at the time when Lord Porter, then Sir George Porter, was Director. In 1989 Phillips moved to Imperial College, London, as Professor of Physical Chemistry, then Head of Department from 1992-2002, and is now Hofmann Professor of Chemistry, and Dean of the Faculties of Life Sciences and Physical Sciences. His address is Department of Chemistry, Imperial College, London SW72AZ, UK; email: d.phillips@ic.ac.uk.
Polymer and Protein Folding Dynamics Revealed By Fluorescence and Infrared Detected Temperature Jump Relaxation

Martin Gruebele
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Large biomolecules such as proteins have the capacity of self-assembly. Assembly can be intermolecular, for example, when many proteins aggregate into an ordered photosynthetic light harvesting antenna complex, or it can be intramolecular, for example, when a single protein acquires its highly packed three-dimensional fold. In recent years, materials and synthetic chemists have also succeeded at creating oligomers capable of folding into very specific structures.

By what dynamical processes does such assembly occur? A chemist with a background in small molecules may be tempted to treat the folding of a single protein as an activated unimolecular reaction $U \rightarrow F$ with one or more steps of single exponential kinetics along a single reaction coordinate. A polymer chemist may envision a collapse not governed by a single rate process, and not describable by a single reaction coordinate. The assembly dynamics of natural and highly ordered artificial polymers lies between these extremes. The formation process often can be described by one or a few exponential functions, and it is slow enough that the picture of an activated rate process is a reasonable approximation. Yet closer examination reveals subtle discrepancies: unusual non-Arrhenius behavior of the rate constant, or different time scales for the kinetics when examined by different spectroscopic probes.

The dynamics corresponding to large-scale structural changes generally ranges from nanoseconds to milliseconds. The lower limit is governed by the time required by macromolecular segments to diffuse through the solvent: a 1000 amu (1kDa) $\alpha$-helix simply does not move over multiple Ångstroms in less than nanoseconds. The upper limit can be much longer than milliseconds, but optimally designed small polymers (<15,000 amu or 15 kDa) generally do not require much more time to settle into a folded structure, or to assemble at sufficiently high monomer concentrations.

One way of investigating such dynamics is by a relaxation experiment. The technique originally was developed by Manfred Eigen in the 1950s, who later was awarded the Nobel prize in Chemistry for this. A small and very rapid perturbation (“the jump”) is applied to the chemical system of interest, such as a change in pH, pressure, or temperature. The equilibrium constant changes abruptly as a result, and the system subsequently relaxes to its new equilibrium. This relaxation can be monitored and yields information about the kinetics of the reaction. Figure 1 illustrates the scheme for a two-state (unimolecular) reaction. This type of approach has several useful features. There is a well-developed branch of nonequilibrium statistical mechanics, linear response theory, which can be used to describe the reaction. When the reaction can be followed in real time, the jump needs to be applied only once: very small amounts of sample are sufficient, in contrast to pump-probe experiments, where the reaction must be initiated many times. Many different probes are suitable for detecting the kinetics; here we discuss near-infrared absorption spectroscopy, and fluorescence emission changes (spectral shifts, lifetime and intensity). Finally, the time response can be quite
fast. For example, water, a common medium for biopolymer reactions, can be heated by an infrared pulse and locally equilibrated in a few picoseconds, long before the polymer chain can respond.

A few years ago we constructed a modern laser version of a temperature jump apparatus to take advantage of these possibilities for folding studies of biopolymers and highly structured artificial polymers. Important dynamics in small proteins and polymers occur on time scales from nanoseconds to milliseconds, and this apparatus allows us to access this full range. A few-microliter water sample containing reactant is heated by a near-infrared pulse from a Raman-shifted Nd:YAG laser. The change in temperature can be calibrated accurately, either by monitoring water transmission at 1.5 \( \mu \)m, or by using a temperature-sensitive fluorescence or absorption probe (such as NATA = N-acetyl tryptophan amide, which mimics the chromophore in many proteins). Subsequent relaxation is monitored by looking at changes in the fluorescence properties or infrared transmission of the sample. For example, when knowledge of the fluorescence spectral shift as a function of time is desired, we can use a multi-channel photomultiplier detector with 200 ns time response to detect fluorescence excited by a train of ultraviolet laser pulses (Figure 2). This yields a snapshot of the full fluorescence spectrum every 200 ns. Looking at such a spectrum, one can ask questions such as: can the time evolution be explained by the linear superposition of two functions (expected for a two-state system), or is it more complicated? Powerful analysis techniques such as singular value decomposition are available to answer such questions.

Consider as an example the folding of a meta-polyphenylethynyl oligomer (Figure 3). This artificial polymer was synthesized in the group of my colleague Jeffrey Moore at the University of Illinois. It consists of phenyl rings bridged by acetylenes in the meta position. By rotating about the acetylene groups, the seventh phenyl ring can be made to superpose on the first in an “all-cis” conformation, forming a “hexagon of hexagons” helical structure. This self-assembly process is highly temperature-sensitive, and can be probed by fluorescence because of the large wavelength red-shift caused when the conjugated groups stack on top of one another and the electronic excitation becomes more delocalized. Wei Yang in my group monitored the kinetics by fast temperature jump relaxation. At a first glance, a simple unimolecular rate description is not unreasonable. The observed relaxation rate has an Arrhenius dependence that would give an activation energy of 77 kJ/mole for the unfolding reaction. Also, the equilibrium spectra as a function of temperature are well described as a superposition of two fluorescence profiles (the “folded” and “unfolded”).

Figure 2. Laser T-jump experiment: A YAG laser is Raman-shifted, then pumps up the temperature of the sample very rapidly. At the same time, a diode-pumped Ti:S laser is frequency tripled to provide UV pulses that probe protein fluorescence every few ns. Detection includes an array photomultiplier to measure spectra, a 500 ps risetime photomultiplier to detect fluorescence lifetimes, and photodiode arrays to detect IR absorption.
This neat picture unravels upon closer examination. The relaxation kinetics contain a continuum of rates in the <10 ns to > 100 ns range. This can be fitted by a stretched exponential of the form Signal(t) = A0 exp[-(t/τ)β], where β is approximately 0.5 at low temperatures. Also, the prefactor that goes with the 77 kJ/mole is 4.1013 µs⁻¹, a completely unrealistic value. As discussed in Figure 1, the observed relaxation rate is not the folding reaction rate, but actually the sum of the folding and unfolding rates (for a simple two-state process). When the equilibrium constant is used to extract an approximate kfold rate constant (ignoring the stretched kinetics for the moment), it exhibits an inverse-Arrhenius dependence. The folding actually slows down slightly as the temperature is raised, and would have a negative activation energy, even under conditions where the folded state is less stable than the unfolded state (above 310 K).

To explain these observations, we treated the oligomers by a lattice statistical mechanical model. Such models are a convenient simplification of the real dynamics. Yet often such simple models can capture the essence of a folding process. In our case, the oligomer was placed on a hexagonal lattice, such that the phenyl rings always lie on the vertices of the hexagons, no matter how the acetylene bridges are isomerized (Figure 3). All conformations of the polymer on the lattice can be enumerated on a computer, and we can use the tools of statistical mechanics and Monte Carlo simulations to reproduce the observed thermodynamics and kinetics. Both types of data are well fit by a stacking energy of about 3.7 kT, an isomerization energy for each acetylene of 0.9 kT, a configurational entropy of kT ln(2.7) per monomer, and a time scale for a single acetylene “flip” of about 5 ps. The thermodynamics of this model comes out looking like a simple two-state system, while the simulated kinetics are stretched exponential with β = 0.5. There is a free energy barrier to folding of 6 kT, but it rapidly increases with temperature, explaining why the observed rate decreases with increasing temperature. The origin of the nonexponential kinetics is the formation on many time scales of partially folded and misfolded states in which either just a few phenyl rings, or the wrong ones, overlap before the correct final structure is reached.

The lesson from these results is that self-assembly of even highly structured polymers is more complicated than a simple two-state chemical reaction, even though superficially they mimic many of the aspects of unimolecular reactions. Yet unlike homopolymers, a very specific structure is formed in the end. The complexities of structured polymer dynamics generally imply that parameters of the simple transition state kinetics picture at least should be renormalized. For instance, it does not make sense to assign a prefactor on the order of femtoseconds or faster to the folding of phenylethynyl oligomers. The shortest relevant time scale is about 5 ps, required for an acetylene bridge to flip around in the solvent. Consequently, the activation barrier is more sensibly set at 15 kJ/mole rather than 77 kJ/mole. It is because of such small activation energies that the temperature dependence of the free energy barrier, usually not an issue in unimolecular chemical reactions, can lead to drastic deviations from Arrhenius behavior, such as activated chemical reactions that slow down when heated, or nonexponential decays. The latter have also been observed during the folding of proteins, presumably because a heterogeneous ensemble of structures is explored by
very fast-folding proteins en route to the folded state. The behavior of the phenylethynylene has also been analyzed successfully by full-atom molecular dynamics simulations, confirming the picture from our simple lattice model.

The concept of a single reaction coordinate is also one that must be carefully examined. Consider the protein folding reaction illustrated in Figure 4. My postdoc Jason Crane and my student Houbi Nguyen collaborated with Jeffery Kelly’s group at the Scripps Institute to examine the folding kinetics of a triple-stranded β-sheet signaling domain, the WW domain (so called because it contains two tryptophans, abbreviated by biochemists as “W”).

In this case, the kinetics are nicely fitted by single exponential decays, but there are some anomalies in the temperature dependence of rate constants when mutants of the protein are examined. In terms of an activated barrier model, this is best understood as follows: We generally expect the reaction rate $k(T)$ to have a weaker temperature dependence than the equilibrium constant $K(T)$. This is a form of Hammond’s principle, originally developed to explain rate trends in organic reactions. The temperature dependence of the reaction rate depends on how the free energy of the transition state changes with temperature; because the transition state has not yet formed all the contacts of the native state, its sensitivity to temperature changes should lie between that of the unfolded and native states of the protein. This is shown in Figure 4. We found that when “mild” mutations (e.g., leucine to isoleucine) are introduced at certain sites, there is no change in the temperature dependence of the reaction. A “stronger” mutation at the same site (e.g., a leucine to alanine mutation in position 7 of the WW domain) causes only a slight decrease in the folding rate, but a much larger temperature dependence, almost like that of the native state. In terms of Hammond’s picture, we would say that the transition state of the mutated protein is only slightly higher in energy than before, but it looks much more like the native state. Thus mutations can change the appearance of the transition state from an “early” one to a “late” one. We can modify the reaction mechanisms by other mutations, and make formation of different structures the rate-limiting step. Thus there is no universal reaction coordinate that describes the folding of all proteins with the WW domain shape. The reason is that what we like to call the transition state is a high-energy plateau in proteins, covered with many smaller minima and saddle points. A certain path through this transition state region may be the lowest in energy, but a mutation or a change in temperature easily can make a different path along different coordinates the lowest in energy. In essence, the protein has many different folding paths programmed into it, increasing robustness to mutations and environmental conditions, and allowing different functions for a fold to evolve in time without disrupting kinetic foldability (Figure 4). Again, one would expect that for such a complicated transition state region, the prefactor of a simple rate model has to be renormalized to include the possibility of hopping around among many small minima. This diffusional hopping leads to prefactors in the nanosecond or even microsecond range, rather than the femtoseconds customary for reactions of small organic molecules.

Figure 4. Left: Folding “hot-spots” of WW domain protein are shown in red. These are residues that have a strong effect on the folding rate and must make side chain contacts in the transition state. Right: “gentle” mutations do not affect the temperature-dependence of the location of the transition state ($\Phi_T = 0$ means the transition state entropy is like the unfolded state, $\Phi_T = 1$ means it is like the native state). More drastic mutations shift the transition state and the folding mechanism: proteins have other channels available to still fold even when stressed.
As a final example, Figure 5 illustrates a multimolecular assembly/disassembly reaction, studied by Hairong Ma from my group and Anjali Pandit from the group of Rienk van Grondelle in the Netherlands.\textsuperscript{10} When the LH1 light harvesting complex of \textit{Rhodospirillum rubrum} is subjected to a temperature jump, its 24-32 subunits disassemble first into dimers, and eventually into monomers. This particular reaction shows a rapid increase of the disassembly rate with temperature, driven by the higher entropy of the disassembling structure. The changes shown in Figure 5 were monitored by infrared absorption of pulses generated by a femtosecond Ti:S laser. However, the time resolution of the experiment is not fs; rather, the mode-locked laser produces a regularly spaced train of IR pulses, which sets the time resolution. The temperature jump heats the sample once, then the pulse train acts as a large number of probe pulses interrogating the sample at regular time intervals. This particular reaction can be described quite well by a simple rate process of the type \( A \rightarrow nB \). Transmission increases at wavelengths corresponding to the fully formed ring (e.g., 873 nm) and decreases at wavelengths corresponding to disassembled dimers of the light harvesting complex (e.g., 820 nm). This particular multi-unit reaction occurs on a millisecond time scale.

To conclude, structure formation or assembly of artificial and biopolymers can be viewed in terms of straightforward kinetic rate models,\textsuperscript{11} but those models should not be pushed too far. Under the surface lurk diffusion processes in high-energy regions of the free energy surface that are much more complicated than what one would expect from small molecule reactions, yet not so random that homopolymer models can be applied. In an activated rate theory, at least the prefactor should be renormalized to reflect the higher dimensionality and roughness of the free energy surface, as well as very low activation energies. Laser-induced relaxation experiments detected by optical probes such as infrared absorption or fluorescence spectroscopy can provide insight into these processes, and yield rate information to test theoretical models and simulations.

References


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Martin Gruebele received his B.S. and Ph.D. degrees from the University of California at Berkeley, before working as a postdoc in the Ahmed Zewail’s group at Caltech. In 1992, he joined the faculty at the University of Illinois, where he now is Alumni Scholar Professor of Chemistry and Professor of Physics, Biophysics and Computational Biology. His group web site can be found at http://garibaldi.nb.uiuc.edu.
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Correction

An incorrect e-mail address was listed for Dr. Steven A. Fleming, author of “Photocyclization” on page 14 of the fall 2002 issue of The Spectrum. Dr. Fleming can be reached by e-mail at steven_fleming@byu.edu.