Photo Isomerization

Isomerization and Dimerization of Indocyanine Green and a Related Heptamethine Dye

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Abstract: Indocyanine green (ICG) and a second heptamethine cyanine dye were studied in detail using 2D NMR spectroscopy at multiple temperatures. In addition to the all-trans conformation, we found in both cases a small percentage of a specific β-γ cis isomer. Exchange rates extrapolated from 2D EXSY spectra under slow exchange conditions are in agreement with that estimated from methine 1H linewidths near coalescence and with previous photo-isomerization studies. We could also confirm our previous hypothesis that, under aerobic conditions in combination with exposure to daylight, ICG undergoes radical dimerization specifically at the γ position.

Introduction

Cyanines are an important class of (mostly synthetic) organic dyes consisting of two nitrogen heterocycles that are connected through a conjugated polymethine chain with an odd number of CH units.[1] The length of the chromophore determines the lowest-energy optical absorption maximum (550–600 nm for trimethines, 600–700 nm for pentamethines and 700–800 nm for heptamethines),[2] and, by adding additional substituents to the heterocycles or the nitrogen atoms, the properties of the dyes can be adapted to multiple applications. While cyanines with unsubstituted methane chains prefer the all-trans conformation as shown by X-ray crystallography[3] and NMR spectroscopy,[4] substitution may lead to a cis conformation of one or several C–C bonds.[5] However, also unsubstituted dyes are long known to undergo trans-cis photo-isomerization following light absorption.[6]

One example of a heptamethine cyanine dye with rapidly growing applications is indocyanine green (ICG, 1, Scheme 1).[7] Due to its sulfonate groups providing excellent water solubility and its very low toxicity[8] ICG is widely used as fluorescent dye.[9] Its recent use in angiography[10] or tumor imaging.[11] In dilute aqueous solution, ICG is not infinitely stable, especially when stored with daylight exposure. However, its stability is significantly increased in concentrated aqueous solutions (where ICG forms J-aggregates) or in organic solvents such as methanol and DMSO.[12] Rotaxane encapsulation has also proved to be an effective tool to stabilize heptamethine dyes against oxidation.[13]

Most conformational studies of cyanines focused on tri- and pentamethine dyes, and, surprisingly, the conformation of ICG itself has never been studied by NMR spectroscopy or X-ray crystallography. In this work, we performed a detailed analysis of the structure and dynamics of the two heptamethine dyes ICG (1) and 1,1′,3,3′,3′-hexamethylindocarbotricyanine iodide (2, Scheme 1) using NMR spectroscopy in solution. We could confirm the all-trans conformation, but also detected a small amount of a specific mono-cis isomer even in the absence of photo-excitation. Furthermore, we present further evidence for the previously proposed[14] dimerization of ICG to 3 in the presence of oxygen and under daylight exposure.

Results and Discussion

Structure of 1 and 2

1 was studied in [D₄]methanol due to the superior stability and larger temperature range compared to water or DMSO, while for the water-insoluble dye 2 we used both [D₄]methanol and CDCl₃. Both dyes have similar lowest-energy optical absorption minima, with a slight blue-shift of 2 in methanol (739 nm) compared to 1 in chloroform (757 nm) and 1 in methanol (786 nm, see Figures S1–S2 in the Supporting Information). Their room temperature 1H NMR spectra are shown in Figure 1.

The spectra are in agreement with two-fold (C₂ᵥ) symmetry, and all 1H, 13C and 15N signals are straightforward assigned using conventional 2D methods (Scheme S1, Table S1). In contrast to the sharp signals of the aromatic head groups and aliphatic side chains, the signals of the methine protons appear broad and can be divided into a group of quite deshielded (β and δ)
For both compounds, strong NOESY correlations are observed between $H^\alpha$ and $H^\delta$, and between $H^\beta$ and $H^{11/12}$. Altogether, NOE-derived distances in 1 agree much better with distances in an all-trans 3D model than with distances in a “$Z\text{E}_{\alpha}Z$” model in which both aromatic head groups are rotated (Table S2). Finally, temperature-dependent proton $T_1$ relaxation times of 1 indicate that only the nitrogen-bonded side chains are flexible within a picosecond/nanosecond timescale and do not fold back to the positively charged backbone (Figure S3).

Isomerization of 1 and 2

We further investigated the intriguing broadening of the methine proton signals by recording spectra at variable temperature. Figure 2 shows the linewidth as function of temperature for the well-resolved protons $H^\alpha$ and $H^\gamma$ of 1 in [D$_4$]methanol at 400 and 800 MHz. Both signals display a local maximum around 298 K with linewidths of approximately 8 Hz (400 MHz) and 14 Hz (800 MHz). Similar maxima (up to 32 Hz for $H^\delta$ in CDCl$_3$ at 800 MHz), albeit at slightly higher temperatures, are found in 2 (Figures S4 and S5). This behavior, which was previously also observed in a trimethine cyanine dye,[16] is typical of a dynamic equilibrium involving a low-populated second species.[17] Indeed, weak ($\approx 3\%$ relative intensity) additional signals appeared in the $^1$H NMR spectra of 2 in CDCl$_3$ near the linewidth minimum at 235 and 248 K. Using NOESY/EXSY spectra at these temperatures (Figure 3), the new signals can be assigned to an isomer with a cis conformation of the $\beta$-$\gamma$ bond, based on the following observations:

- The signals show exchange with that of the main (all-trans) isomer, with two exchange peaks each for $H^\alpha$, $H^\beta$ and $H^\gamma$ (in Figure 3 highlighted in purple for $H^\beta$). Using COSY (Figure S7) and $^1$H,$^1$C-HSQC (Figure S8), all methine $^1$H and $^1$C resonances of the minor isomer could be assigned (Table S3).

Figure 1. $^1$H NMR spectra of 1 in [D$_4$]methanol (top), 2 in [D$_4$]methanol (middle) and 2 in CDCl$_3$ (bottom), recorded at 400 MHz and 298 K. Only methine, side chain and methyl groups are labelled.

Scheme 1. Chemical formulae of 1, 2 and 3 with the positions numbered for NMR assignment.

and a group of more shielded ($\alpha$ and $\gamma'$) positions.[15] All methine protons show exclusively trans coupling constants ($^3J_{\text{trans}} \approx 13$ Hz).
One neighboring Hβ/Hγ pair shows a relatively strong NOE correlation with a doublet of doublets $^3J_{\text{trans}} \approx 13 \text{ Hz}$, $^3J_{\text{cis}} \approx 9 \text{ Hz}$ fine structure (Figure S6), indicating these protons are in a cis position.

The adjacent protons Hα and Hδ likewise show a strong NOE correlation (green circle in Figure 3).

The observed chemical shift trends qualitatively reflect the positions of the protons relative to the kinked methine chain (Hβ, Hγ upfield = convex, Hα, Hδ downfield = concave side).[16]

Peak overlap makes the analogous analysis of 1 in [D₄]methanol substantially more challenging, but we could identify the same β-γ cis isomer with the same chemical shift trends (Figures S9–S11, Table S5). In contrast (and partially due to the poor solubility at low temperatures), no exchange with a minor isomer was found for compound 2 in [D₄]methanol. Table 1 summarizes the thermodynamic parameters for the β-γ isomerization obtained from the NOESY/EXSY spectra at 235 and 248 K and extrapolated to ambient temperature. The extrapolated values are in good agreement with the linewidths of the Hα and Hγ signals (see Supporting Information).

For 1, the values are also in reasonable agreement with previous reports of a (not further specified) cis photo-isomer of 1·NaI in methanol with a (dark) steady-state population of 6.2 % and a lifetime of 430 μs (corresponding to $k_{\text{trans-cis}} = 154 \text{ s}^{-1}$).[18]
For the photo-isomer of 2, a similar lifetime of 300 µs was reported in DMSO.[19] For this dye, it was also shown that \textit{trans-cis} isomerization depends on both solvent and counterion,[20] which may be explained by the positive charge of the poly-methine chain being more localized in the twisted transition state geometry.[21] Presumably, in our case, \textit{cis-trans} isomerization of 2 in highly polar DMSO is too fast to be observable by NMR spectroscopy at 235 K. In less polar CDCl₃ it is slowed down but at the same time affected by ion-pair formation, while isomerization in 1 is slowed down by the bulkier head groups.

Surprisingly, neither for 1 nor for 2 we could identify further isomers with \textit{cis} conformation at other positions of the methine chain.[22] Gas phase DFT calculations of 2 suggest similar or even lower energy and barriers for the 10-\(\alpha\) and the \(\gamma\)-\(\delta\) \textit{cis} isomers, while the \(\alpha\)-\(\beta\) \textit{cis} isomer is sterically highly congested (Table S4 and ref.[23]). Isomerization barriers of cyanines calculated ab initio in the gas phase have generally been found too high,[24] and more realistic values, again very similar for all bonds, have been obtained in a recent semi-empirical study.[25]

In our case, we cannot exclude the presence of further isomers of 1 and 2 in solution, but the \(\beta\)-\(\gamma\) isomer is certainly distinct by several kJ/mol in terms of relative stability and/or isomerization barrier. In order to reproduce these subtle effects, more sophisticated calculations with inclusion of solvent will have to be performed in the future.

**Structure and Formation of 3**

In a previous report, the stability of 1 was studied in aqueous solution, and a dimeric species 3 could be identified by DOSY NMR as the dominant degradation product.[14] Further NMR experiments suggested that the two monomeric fragments in 3 are connected through the \(\gamma\) carbons of the methine chain. Here, we present further evidence for the \(\gamma\)-\(\gamma\) dimer structure of 3 using a chromatographically isolated sample:

- The barely shifted lowest-energy absorption maximum of 3 (778 nm vs. 786 nm in 1, Figure S1) in methanol indicates that the \(\pi\)-system remains intact.
- Singly (Figure S13) and doubly (Figure S14) charged ions in the ESI(–) mass spectrum of 3 confirm the anticipated mass of 1502.57 g/mol.
- All \(1^H, 13^C\) and \(15^N\) resonances of 3 in [D₄]methanol could be assigned (Table S6). There are only 6 methine proton signals, 3 in the upfield (\(\alpha\), \(\gamma\), \(\alpha\)' Figure S14) and 3 in the downfield (\(\beta\), \(\delta\), \(\beta\)', Figure S14) region. No proton signal is visible for the bridging \(\gamma\) position, and the neighboring (\(\beta\), \(\delta\)) protons now appear as doublets (\(J_{\beta\delta} \approx 13\) Hz).
- 4 distinct signals for the methyl groups indicate that 3 is not planar, but the two methine chains are twisted with respect to each other, forming a short (\(\alpha\), \(\beta\)) and a long (\(\delta\), \(\beta\)', \(\alpha\)') arm. Inter-monomer NOE correlations indicate a twist of about 90° (Figures S16–S17, Table S7), in agreement with the crystal structure of a chemically synthesized pentamethine \(\gamma\)-\(\gamma\) dimer, where a twist of \(\approx 65°\) was found.[26]

Interestingly, we also found traces of 3 in an aged sample of 1 in [D₄]methanol. Since dimerization corresponds to an oxidation of the dye under formal loss of H₂, we prepared three NMR samples in [D₄]methanol: one was saturated with oxygen and exposed to daylight, one sample was saturated with oxygen, but kept in the dark, while the last sample was degassed and sealed, but again exposed to daylight. Only in the first sample we noted formation of 3 (≈ 50 % after 16 days, Figure 4), while the samples that were degassed or kept in the dark showed no sign of 3 even after several weeks (Figure S18).

The results show that both oxygen and light are required for the formation of 3. We therefore propose a mechanism that involves single electron transfer to singlet oxygen, followed by attack of the intermediate radical (cation) on a second dye molecule, loss of a second electron, and deprotonation (Scheme 2).

Scheme 2. Proposed mechanism for the formation of 3.

Alternatively, two radicals may form independently and recombine, as it has been proposed for a series of related pentamethine dyes.[27] In this study, it was shown that radicals form reversibly under very mild (= +0.7 V) electrochemical or chemical conditions. The subsequent irreversible dimerization occurred preferentially at the positions \(\alpha\)-\(\gamma\) or \(\gamma\)-\(\gamma\), depending on the steric requirements of the head groups. Singlet oxygen is easily generated in situ by photo-sensitization from the cyanine dye,[28] in agreement with our observation that 3 also forms under illumination with a red LED as light source. The negatively charged side groups in 1 make the overall net charge of the dye radical zero and, hence, possibly facilitate the dimeriza-
tion. This would explain our observation that also 2 forms γ-γ dimers, but at a much slower rate and with several side products including α-OH and γ-OH species.

Conclusion

In summary, we could show that both 1 and 2 exist in solution as the main all-trans isomer in equilibrium with a small amount of at least one cis isomer (β-γ). Exchange between the isomers is responsible for broadening of the methine 1H signals around room temperature. In the presence of oxygen and daylight, 1 specifically forms γ-γ dimers (3) via a radical mechanism.

Experimental Section

1 and 2 were commercially obtained from Sigma Aldrich. 3 was obtained from an aqueous solution and isolated by reversed-phase HPLC according to reference 14. [D₄]Methanol and CDCl₃ were purchased from Deutero GmbH. All chemicals were used without further purification.

For NMR measurements, 10 mg of 1 and 1.5 mg of 3 were dissolved in 0.6 mL of [D₄]methanol. For measurements of 2, 10 mg were dissolved in CDCl₃ for 1D NMR experiments, 30 mg in CDCl₃ for the 2D experiments and 8 mg in [D₄]methanol (maximum solubility). All NMR samples were stored at 3 °C. For the in-situ monitoring of the dimerization of ICG three samples were prepared, each containing 10 mg of dissolved in 0.6 mL of [D₄]methanol of which two were saturated with oxygen through a cannula. One sample was stored at room temperature at light exposure, a second one was kept in the dark at the same temperature. The third sample was degassed and kept under N₂ atmosphere, but again exposed to light.

NMR spectra were recorded on a Bruker Avance III HD 400 MHz spectrometer equipped with a 5 mm BBI probe, a Bruker Avance III HD 500 MHz spectrometer equipped with a 5 mm BBO cryo probe, a Bruker Avance NEO HD 800 MHz spectrometer equipped with a 3 mm TCI cryo probe (low temperature limit 235 K) and a Bruker Avance III HD 800 MHz spectrometer equipped with a 3 mm TCI cryo probe (low temperature limit 260 K). All spectra were, if not differently indicated, recorded at 298 K. The spectra were referenced to the residual solvent signal (CDCl₃: δ(H) = 7.26 ppm, δ(C) = 77.16 ppm). ¹⁵N chemical shifts were indirectly referenced using the lock signal and nitromethane as a standard. NMR spectra were processed with TopSpin 3.2 and analyzed with MestReNova 10.

Proton T₁ relaxation times were obtained using the t1ir (inversion recovery) sequence with recovery delays of 1, 3, 10, 30, 100, 300, 1000, 3000 ms and the TopSpin T1T2 module for analysis. NOESY spectra at 298 K for the conformational analysis of 1 and 3 were acquired with mixing times of 500 and 250 ms, respectively. In both cases, all cross peaks were < 5% of the diagonal intensities, indicating the mixing times are in the linear buildup region. EXSY spectra were recorded at temperatures of 235 and 248 K with mixing times of 500, 50 and 5 ms. The diagonal and cross signals belonging to H² (1) and H³ (2) were used for integration, exchange rates were determined from the integrals with EXSYCalc (MestReLab-Research). 3D models for the distance measurements were created with Chem3D 16.0.

DFT calculations were carried out with Gaussian 09[29] using the B3LYP method and the basis set 6-31+g(d). For ground state structures, standard optimization and frequency calculations were performed. Transition state structures were calculated using Berry optimization and the QST3 method. Mass spectra were recorded in methanol on a Bruker maXis ESI QTOF spectrometer using standard ESI(−) parameters. UV/Vis spectra were recorded in methanol and in chloroform on a Varian Cary 50 and an Agilent Technologies Cary 300 UV/Vis spectrometer.

Acknowledgments

We thank the group of Prof. Dr. Griesinger at the MPI-BPC for 800 MHz spectrometer time and Dr. Frauentor for measuring mass spectra. In addition, we acknowledge the group of Prof. Dr. Schneider for allowing us to use the UV/Vis spectrometer and especially Richt van Alten for help with the measurements.

Keywords: Cyanines · Isomerization · Dimerization · NMR spectroscopy · Conformation analysis

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