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# Absorption and Emission Spectroscopic Investigation of the Thermal Dynamics of the Archaerhodopsin 3 Based Fluorescent Voltage Sensor QuasAr1

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**Abstract:** QuasAr1 is a fluorescent voltage sensor derived from Archaerhodopsin 3 (Arch) of *Halorubrum sodomense* by directed evolution. Here we report absorption and emission spectroscopic studies of QuasAr1 in Tris buffer at pH 8. Absorption cross-section spectra, fluorescence quantum distributions, fluorescence quantum yields, and fluorescence excitation spectra were determined. The thermal stability of QuasAr1 was studied by long-time attenuation coefficient measurements at room temperature ( $23 \pm 2$  °C) and at  $2.5 \pm 0.5$  °C. The apparent melting temperature was determined by stepwise sample heating up and cooling down (obtained apparent melting temperature:  $65 \pm 3$  °C). In the protein melting process the originally present protonated retinal Schiff base (PRSB) with absorption maximum at 580 nm converted to de-protonated retinal Schiff base (RSB) with absorption maximum at 380 nm. Long-time storage of QuasAr1 at temperatures around 2.5 °C and around 23 °C caused gradual protonated retinal Schiff base isomer changes to other isomer conformations, de-protonation to retinal Schiff base isomers, and apoprotein structure changes showing up in ultraviolet absorption increase. Reaction coordinate schemes are presented for the thermal protonated retinal Schiff base isomerizations and deprotonations in parallel with the dynamic apoprotein restructurings.

**Keywords:** QuasArs; Archaerhodopsin 3; genetically encoded voltage sensors (GEVIs); absorption spectroscopic characterization; fluorescence spectroscopic characterization; apparent protein melting temperature; thermal stability; thermal isomerization; thermal deprotonation

## 1. Introduction

Changes in electrical potential across the plasma membrane of neurons are important for intercellular and intracellular signal transmission [1]. Classical electrophysiology techniques involve placing electrodes into biological tissue allowing to record membrane currents [2,3]. Optical recordings of membrane potential from cells, especially neurons, with fluorescent voltage sensitive dyes [4–8], genetically encoded calcium indicators (GECIs) [9–14], and with fluorescent genetically encoded voltage indicators (GEVIs) [15–28] is an active field of research. Two major groups of GEVIs are i) integral membrane voltage sensing domains (VSDs) composed of four trans-membrane helices fused to fluorescent proteins [15,17–20,25–27,29–34] and ii) microbial rhodopsins composed of 7 trans-membrane  $\alpha$ -helices with covalently bound retinal isomers [14,16,25–27,35–38]. Generally microbial rhodopsins exhibit low fluorescence quantum yield in the range of  $\phi_F = 2 \times 10^{-4}$  to  $10^{-5}$  [39–41] which is too low for any cellular application. Directed evolution approach yielded modified microbial rhodopsins with increased fluorescence quantum yield, and few of them exhibited change of the fluorescence

intensity depending on the membrane voltage [14,15,25–27,35–38,42–48]. In rhodopsin-fluorescent protein GEVIs a microbial rhodopsin is fused with a highly fluorescent protein and the emission of the fused fluorescent protein changes upon membrane voltage changes [16,28,49,50].

Archaerhodopsin 3 (Arch) from *Halorubrum sodomense* with a single residue mutation D95N showed potential as a GEVI [36] and initiated the development of the Arch variants QuasAr1 and QuasAr2 (named according to ‘Quality superior to Arch’) [42]. QuasArs have improved fluorescence intensity and membrane voltage sensitivity [22,42,45]. The fluorescence quantum yield of wild-type Arch was reported to be  $\phi_F = 9 \times 10^{-4}$  [36], of the mutant Arch D95N it was  $\phi_F = 4 \times 10^{-4}$  [36,42], while for QuasAr1 it increased to  $\phi_F = 8 \times 10^{-3}$  [42], and for QuasAr2 it was found to be  $\phi_F = 4 \times 10^{-3}$  [42]. The lower fluorescence quantum yield of QuasAr2 compared to QuasAr1 is compensated by its higher voltage sensitivity [42]. QuasAr1 differs from the wild-type Arch by 5 mutations, namely P60S, T80S, D95H, D106H, and F161V [42]. The amino acid sequence of QuasAr1 is shown in Figure S1 of the Supplementary Materials (Section S1). Some structural formulae of retinal cofactors of rhodopsins are found in [51] and are shown in Figure S2 of the Supplementary Materials (Section S2). QuasAr2 differs from QuasAr1 by the counter ion mutation H95Q.

Here a detailed study is presented of the absorption and emission spectroscopic properties and the thermal dynamics of QuasAr1 in pH 8 Tris buffer. Aliquots of 30  $\mu$ L were used in the studies. They were stored at  $-80^\circ\text{C}$  and thawed before usage. The absorption cross-section spectrum, excitation wavelength dependent fluorescence emission quantum distributions and quantum yields, and emission wavelength dependent fluorescence excitation spectra of purified QuasAr1 were determined. The thermal stability of QuasAr1 was studied by long-time spectroscopic studies at room temperature (21–25  $^\circ\text{C}$ ) and refrigerator temperature of  $2.5 \pm 0.5^\circ\text{C}$ . The apparent melting temperature was determined by stepwise sample heating up and cooling down. The temperature and time dependent retinal chromophore and opsin protein changes are discussed.

## 2. Results

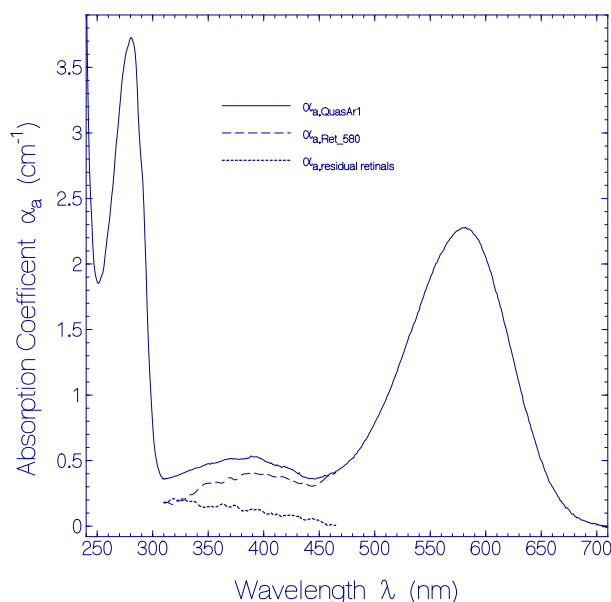
### 2.1. Absorption and Emission Behavior of Fresh Thawed QuasAr1 Samples

The absorption coefficient spectrum  $\alpha_a(\lambda)$  of a fresh thawed QuasAr1 sample was measured after centrifugation with 4400 rpm for 30 min at  $4^\circ\text{C}$  (Centrifuge 5702 R, Eppendorf AG, Hamburg, Germany). It is displayed by the solid curve in Figure 1. The main absorption band with maximum at wavelength  $\lambda \approx 580$  nm is attributed to the singlet  $S_0$ – $S_1$  transition of protonated retinal Schiff base (PRSB) and named Ret\_580. The absorption in the range from 310 nm to 465 nm is thought to be comprised of singlet  $S_0$ – $S_n$  ( $n \geq 2$ ) transitions of Ret\_580 (dashed curve  $\alpha_{a,\text{Ret}_580}(\lambda)$  in Figure 1, for its determination see section S3 of the Supplementary Materials) and singlet ground-state–excited-state transitions of residual retinal components (dotted curve  $\alpha_{a,\text{residual retinals}}(\lambda) = \alpha_{a,\text{QuasAr1}}(\lambda) - \alpha_{a,\text{Ret}_580}(\lambda)$  in Figure 1). The short-wavelength absorption band peaking at  $\lambda = 280$  nm is determined by apoprotein absorption (Trp, Tyr, Phe) and some retinal contribution.

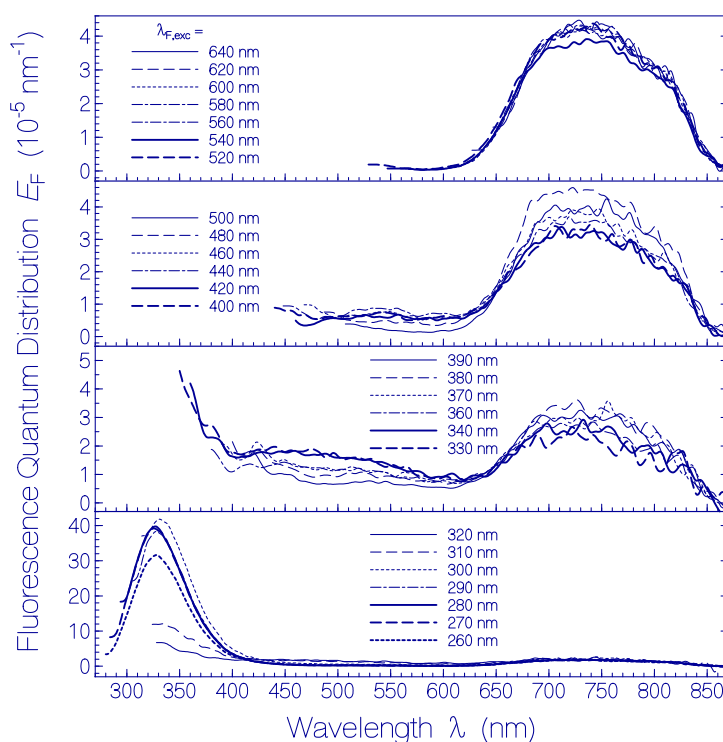
The absorption cross-section spectrum of Ret\_580, i.e.,  $\sigma_a(\lambda) = \alpha_{a,\text{Ret}_580}(\lambda) / N_{\text{Ret}_580}$  where  $N_{\text{Ret}_580}$  is the number density of Ret\_580 chromophores in QuasAr1, is determined in section S3 of the Supplementary Materials (Figure S3).

Fluorescence emission quantum distributions  $E_F(\lambda)$  of a fresh thawed QuasAr1 sample in pH 8 Tris buffer for fluorescence excitation wavelengths  $\lambda_{F,\text{exc}}$  in the range from 260 nm to 640 nm are shown in Figure 2 and the corresponding fluorescence quantum yields are included in Figure 3 (see below). For  $\lambda_{F,\text{exc}} > 500$  nm only  $S_1$ – $S_0$  emission from Ret\_580 is observed. The wavelength position of peak fluorescence emission is at  $\lambda_{F,\text{max}} \approx 740$  nm. The full spectral half-width of this emission is  $\delta\tilde{\nu}_{F,\text{Ret}_580} \approx 2900$   $\text{cm}^{-1}$ . The Stokes shift is  $\delta\tilde{\nu}_{\text{Stokes}} = \lambda_{a,\text{max}}^{-1} - \lambda_{F,\text{max}}^{-1} \approx 3730$   $\text{cm}^{-1}$ . The strong Stokes shift and broad spectral width of the Ret\_580 emission spectra indicate fluorescence emission along the  $S_1$  excited state photo-isomerization path. Below it will be shown that Ret\_580 is composed dominantly of two protonated retinal Schiff base isomers (Ret\_580<sub>I</sub> and Ret\_580<sub>II</sub>) in

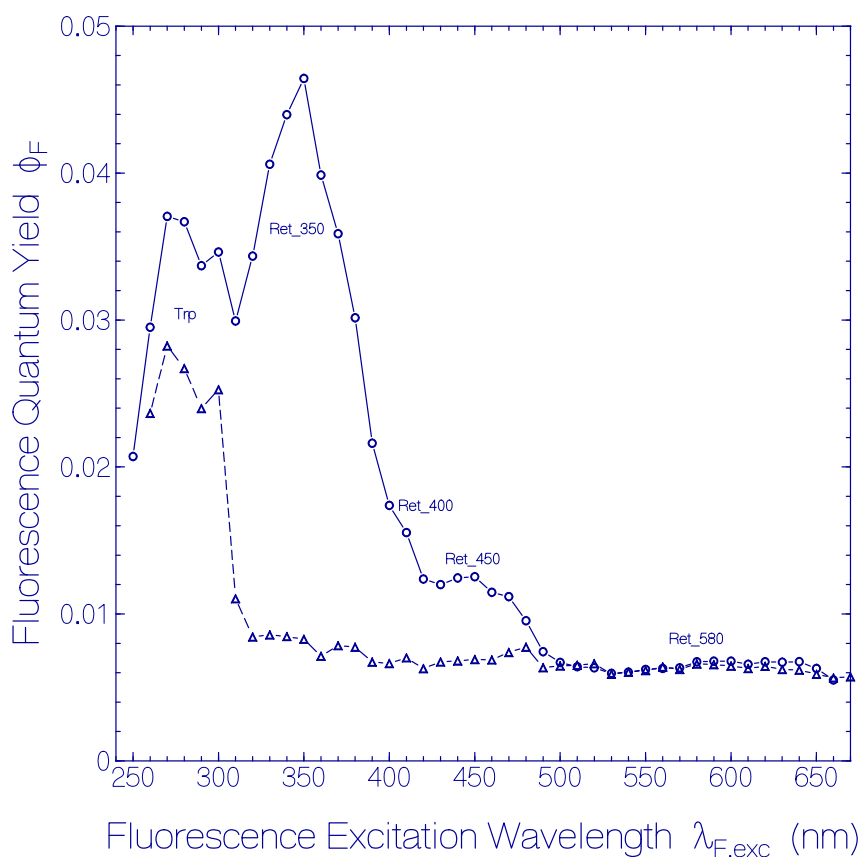
different apoprotein conformations (Apoprotein<sub>I</sub> and Apoprotein<sub>II</sub>) which contribute to the broad fluorescence emission. In the Supplementary Materials (Table S1) wavelength positions of absorption maxima and fluorescence maxima together with Stokes shifts are listed for several rhodopsins (range of Stokes shifts from  $\delta\tilde{\nu}_{Stokes} \approx 1750 \text{ cm}^{-1}$  for histidine kinase rhodopsin 1 from *Chlamydomonas reinhardtii* to  $\delta\tilde{\nu}_{Stokes} \approx 4900 \text{ cm}^{-1}$  for proteorhodopsin from uncultivated marine  $\gamma$ -proteobacteria).



**Figure 1.** Absorption coefficient spectrum of a fresh thawed QuasAr1 sample in pH 8 Tris buffer. Solid curve: measured absorption coefficient spectrum  $\alpha_{a,QuasAr1}(\lambda)$ . Dashed curve: absorption coefficient spectrum  $\alpha_{a,Ret\_580}(\lambda)$  of PRSB Ret\_580. Dotted curve: absorption coefficient spectrum of residual retinal components  $\alpha_{a,residual\ retinals}(\lambda) = \alpha_{a,QuasAr1}(\lambda) - \alpha_{a,Ret\_580}(\lambda)$ .



**Figure 2.** Fluorescence emission quantum distributions  $E_F(\lambda)$  of fresh thawed QuasAr1 in pH 8 Tris buffer. The fluorescence excitation wavelengths  $\lambda_{F,exc}$  are indicated in the sub-figures.



**Figure 3.** Dependence of the total fluorescence quantum yield  $\phi_F$  on the fluorescence excitation wavelength  $\lambda_{F,exc}$  for QuasAr1 in pH 8 Tris buffer. The dashed line connected triangles belong to a fresh thawed sample. The line-connected circles belong to a sample stored at  $2.5 \pm 0.5$  °C in the dark over a period of 80 days.

Fluorescence excitation in the wavelength range from 400 nm to 480 nm indicates an additional fluorescence emission band around  $\lambda_{F,max} \approx 550$  nm. It is thought to be caused by a small amount of a protonated retinal Schiff base isomer (named Ret\_450, see below) absorbing in this range ( $\delta\tilde{\nu}_{Stokes} \approx 4000$   $\text{cm}^{-1}$ ). Fluorescence excitation in the range from 330 nm to 390 nm resulted in a broad fluorescence emission band around  $\lambda_{F,max} \approx 470$  nm. It is attributed to fluorescence emission of deprotonated retinal Schiff base isomer components. For fluorescence excitation in the wavelength region from 260 nm to 320 nm the fluorescence emission is dominated by Trp emission of the QuasAr1 apoprotein. The fluorescence emission maximum occurs at  $\lambda_{F,max} = 328$  nm ( $\delta\tilde{\nu}_{F,Trp} \approx 5600$   $\text{cm}^{-1}$ ,  $\delta\tilde{\nu}_{Stokes} \approx 5200$   $\text{cm}^{-1}$ ). For all excitation wavelengths, the Ret\_580 fluorescence emission band around  $\lambda_{F,max} = 740$  nm is present since the Ret\_580 absorption extends over the whole applied fluorescence excitation wavelength region due to  $S_0$ - $S_n$  transitions ( $n \geq 1$ ) with fast  $S_n$ - $S_1$  nonradiative relaxation for  $n \geq 2$  and  $S_1$ - $S_0$  radiative emission. Additionally Förster-type energy transfer [52,53] occurs from Tyr and Trp to Ret\_580 in the case of Tyr and Trp photo-excitation.

The dependence of the total fluorescence quantum yield  $\phi_F = \int_{em} E_F(\lambda) d\lambda$  (the integration runs over the whole fluorescence emission wavelength region) of fresh thawed QuasAr1 on the fluorescence excitation wavelength  $\lambda_{F,exc}$  is depicted by the dashed curve connected triangles in Figure 3. The fluorescence quantum yield is  $\phi_F = (6.5 \pm 0.5) \times 10^{-3}$  for excitation in the wavelength region of  $S_0$ - $S_1$  absorption of Ret\_580 ( $\lambda_{F,exc} \geq 490$  nm). In the range of  $380 \text{ nm} \leq \lambda_{F,exc} \leq 480$  nm it is  $\phi_F = (7 \pm 0.5) \times 10^{-3}$ , and in the range  $320 \text{ nm} \leq \lambda_{F,exc} \leq 380$  nm it is  $\phi_F = (8 \pm 0.5) \times 10^{-3}$  indicating a somewhat increased fluorescence efficiency of the additionally present retinal species besides Ret\_580. In the range of  $260 \text{ nm} \leq \lambda_{F,exc} \leq 310$  nm the fluorescence emission is dominated

by apoprotein Trp emission. There the fluorescence quantum yield increased to  $\phi_F = 0.026 \pm 0.002$ . The fluorescence emission of photo-excited Tyr is quenched by Förster-type energy transfer [52,53] to Trp (see supplementary material to [54]). The Trp fluorescence is reduced by Förster-type energy transfer [52,53] from Trp to the retinals in QuasAr1. The fluorescence quantum yields of Tyr and Trp in neutral water at 20 °C are  $\phi_F(\text{Tyr}) = 0.14$  [55] and  $\phi_F(\text{Trp}) = 0.15$  [56,57].

Normalized fluorescence excitation quantum distributions  $E'_{ex}(\lambda)$  of a fresh thawed QuasAr1 sample in pH 8 Tris buffer for fluorescence detection wavelengths  $\lambda_{F,det}$  in the range from 300 nm to 780 nm are shown in Figure S4 of the Supplementary Materials (section S4). They confirm the excitation wavelength dependent fluorescence emission of the dominant retinal component Ret\_580 and other present retinal components.

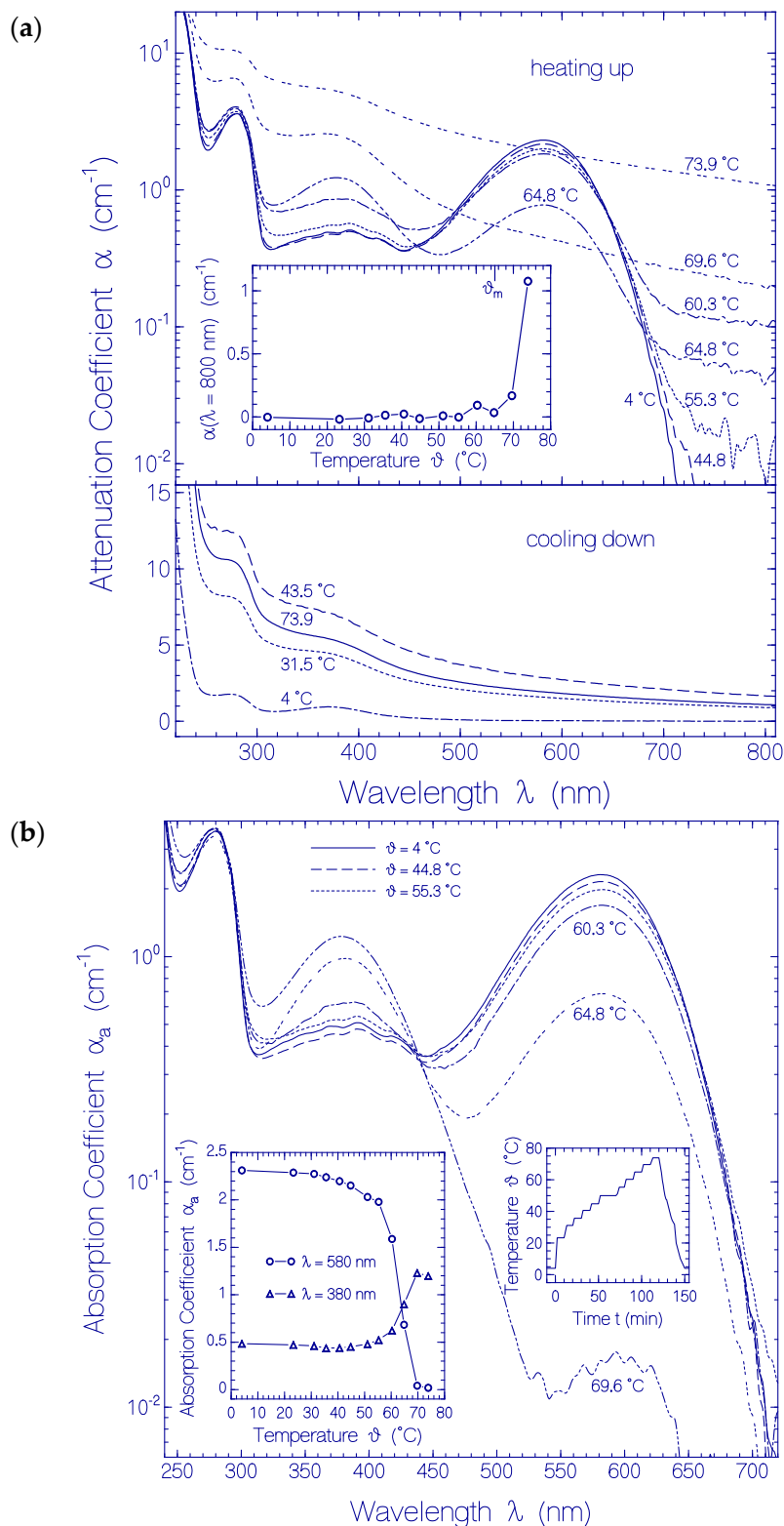
## 2.2. Heating-Cooling Cycle of a Fresh Thawed QuasAr1 Sample

A fresh thawed sample of QuasAr1 was stepwise heated up to  $\vartheta = 73.9$  °C, and then cooled down. Thereby attenuation coefficient spectra were measured. The results are shown in Figure 4. The applied temporal heating and cooling temperature profile is depicted in the right inset of Figure 4b. The apparent QuasAr1 protein melting temperature  $\vartheta_m$  was determined by the onset of a steep attenuation rise in the transparency spectral region of QuasAr1 [58] due to coalescing of denatured unfolded proteins [59]. The apparent protein melting temperature is an indicator of the protein thermal stability.

The temperature dependent development of attenuation coefficient spectra  $\alpha(\lambda)$  of QuasAr1 is shown in Figure 4a. Up to about 55 °C the attenuation spectra remained nearly unchanged (see top part of Figure 4a). Then the attenuation band of Ret\_580 decreased and a new attenuation band around 380 nm built up (Ret\_380). Light scattering became detectable above 55 °C and increased strongly above 65 °C. The temperature dependence of the light attenuation in the transparency region of QuasAr1 at 800 nm during the sample heating up is shown in the inset of the top part of Figure 4a. The apparent protein melting temperature determined by the onset of steeply rising light attenuation (light scattering) is  $\vartheta_m = 65 \pm 3$  °C. The light scattering increased during heating up to 73.9 °C, and continued to increase during cooling down to 43.5 °C (see bottom part of Figure 4a). Then the light attenuation decreased likely due to aggregated particle sedimentation (see attenuation curve belonging to 31.5 °C in bottom part of Figure 4a). The final attenuation curve (dash-dotted curve in bottom part of Figure 4a) was obtained after centrifugation of the sample for 20 min with 4400 rpm at 4 °C.

The main part of Figure 4b shows the temperature dependent development of absorption coefficient spectra  $\alpha_a(\lambda)$  of QuasAr1 during stepwise sample heating up (the attenuation coefficient spectra of Figure 4a were deprived of their scattering contribution, see procedure described in Section 4.2). The PRSB Ret\_580 absorption band peaking around 580 nm decreased with rising temperature by deprotonation to RSB Ret\_380 forming a new absorption band around 380 nm. For  $\vartheta = 69.6$  °C Ret\_580 is nearly completely converted to Ret\_380. Therefore the curve  $\alpha_a(\lambda, \vartheta = 69.6$  °C) in the wavelength range from  $\approx 310$  nm to  $\approx 500$  nm of Figure 4b represents the absorption coefficient spectrum of Ret\_380. The absorption cross-section spectrum of Ret\_380 is determined in section S3 of the Supplementary Materials from  $\alpha_a(\lambda, \vartheta = 4$  °C) and  $\alpha_a(\lambda, \vartheta = 69.6$  °C) of Figure 4b (dashed curve in Figure S3).

The left inset in Figure 4b displays the temperature dependent development of the absorption coefficients  $\alpha_a(\vartheta)$  at  $\lambda = 580$  nm (line-connected circles) and at  $\lambda = 380$  nm (line-connected triangles). The curves clearly show the rising conversion of Ret\_580 to Ret\_380 with increasing temperature. The absorption at 380 nm below 40 °C is determined by the  $S_0$ - $S_n$  absorption of Ret\_580 and the absorption of the already present deprotonated retinal Schiff base isomers of the fresh thawed unheated sample. In the stepwise sample heating the conversion of PRSB (Ret\_580) to RSB (Ret\_380) starts already at about  $\vartheta = 40$  °C and becomes very strong above  $\vartheta = 55$  °C well below the apparent protein melting temperature of  $\vartheta_m \approx 65$  °C.

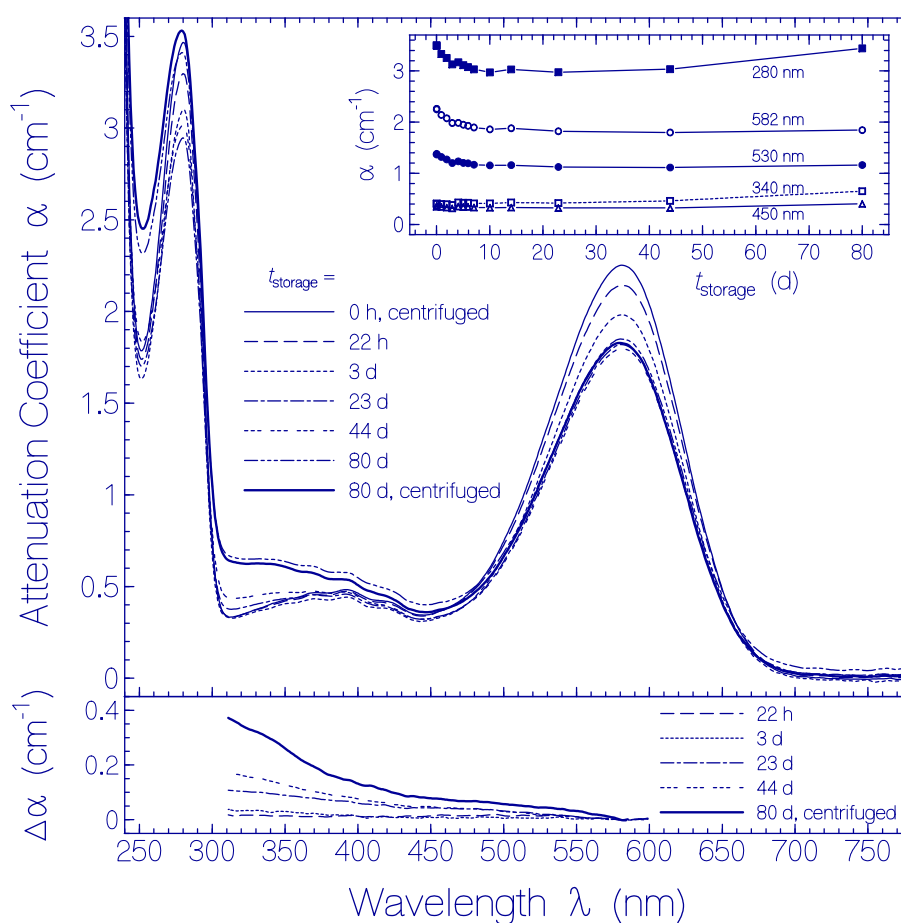


**Figure 4.** Heating-cooling cycle behavior of a fresh thawed QuasAr1 sample in pH 8 Tris buffer. (a) Attenuation coefficient spectra  $\alpha(\lambda)$  development during stepwise sample heating up (top part) and cooling down (bottom part). Inset in top part: Temperature dependent attenuation coefficient development  $\alpha(800 \text{ nm})$  during sample heating up. (b) Absorption coefficient spectra  $\alpha_a(\lambda)$  development during stepwise sample heating up. Left inset: Temperature dependent absorption coefficient development  $\alpha_a(580 \text{ nm})$  and  $\alpha_a(380 \text{ nm})$ . Right inset: Applied heating and cooling temperature profile  $\vartheta(t)$ .

### 2.3. Temporal Development of QuasAr1 at Refrigerator Temperature of 2.5 °C

The thermal stability of QuasAr1 in pH 8 Tris buffer at  $\vartheta = 2.5 \pm 0.5$  °C in the dark was studied by carrying out transmission spectra measurements over a duration of 80 days and by measuring fluorescence emission and fluorescence excitation spectra at the end of the storage time.

The temporal development of the attenuation coefficient spectra  $\alpha(\lambda)$  is shown in Figure 5. In the top main part attenuation coefficient spectra are shown for selected storage times  $t_{\text{storage}}$ . For  $t_{\text{storage}} = 80$  days the attenuation spectra are shown before and after sample centrifugation to see the small light scattering contribution due to protein aggregation. The top inset shows the temporal attenuation coefficient development for some selected wavelengths. The bottom part displays difference attenuation spectra  $\Delta\alpha(\lambda, t_{\text{storage}}) = \alpha(\lambda, t_{\text{storage}}) - \alpha(\lambda, 0) \left[ \alpha(580 \text{ nm}, t_{\text{storage}}) / \alpha(580 \text{ nm}, 0) \right]$  for selected storage times.



**Figure 5.** Temporal development of attenuation coefficient spectra  $\alpha(\lambda, t_{\text{storage}})$  of QuasAr1 in pH 8 Tris buffer at  $2.5 \pm 0.5$  °C in the dark. The storage times in the refrigerator are listed in the legend. The top inset shows attenuation coefficients at fixed wavelengths versus the storage time  $t_{\text{storage}}$ . The bottom part displays difference attenuation spectra  $\Delta\alpha(\lambda, t_{\text{storage}}) = \alpha(\lambda, t_{\text{storage}}) - \alpha(\lambda, 0) \left[ \alpha(580 \text{ nm}, t_{\text{storage}}) / \alpha(580 \text{ nm}, 0) \right]$ .

Within the first ten days the attenuation coefficient of Ret\_580 around 580 nm and the attenuation coefficient of the apoprotein around 280 nm decreased with time while the attenuation in the range from 310 nm to 470 nm remained approximately unchanged. The attenuation reduction around 580 nm and around 280 nm are attributed i) partly to the conversion of Ret\_580 to other retinals absorbing in the 470–310 nm range (see differential attenuation coefficient spectra in the bottom part of Figure 5) and ii) partly to QuasAr1 tight small aggregate formation (specific surface reduction [60–62])

and/or loose aggregate cluster compactization with storage time (cluster size small therefore not showing up in light attenuation in the transparency spectral region; loosely packed globules with small volume fill factor densify to tightly packed globules, thereby the apparent absorption cross-section per molecule decreases because of specific surface reduction of the aggregates [63]). In the time range from  $t_{\text{storage}} = 10$  d to 80 d the Ret\_580 absorption band remained nearly unchanged. However, in this time range the apoprotein absorption changed. The main apoprotein absorption band around 280 nm increased and broadened (stronger absorption around 250 nm and around 310 nm). This behavior is attributed to dynamic QuasAr1 apoprotein restructuring. The increase of the apoprotein absorption strength is attributed to some increase of the oscillator strength of the  $S_0$  to  $S_1$  transition of Trp due to protein restructuring. In the wavelength range from 310 nm to 400 nm some absorption contribution from possibly formed dityrosine [64], tyrosinyl radicals [65], and tryptophanyl radicals [66] cannot be excluded [67]. The tryptophan involvement as chromophore element in photoreceptors is known for the UV-B photoreceptor UVR8 from *Arabidopsis thaliana* [68,69] (see also [51] with references therein) and for the LITE-1 photoreceptor in *Caenorhabditis elegans* [70,71].

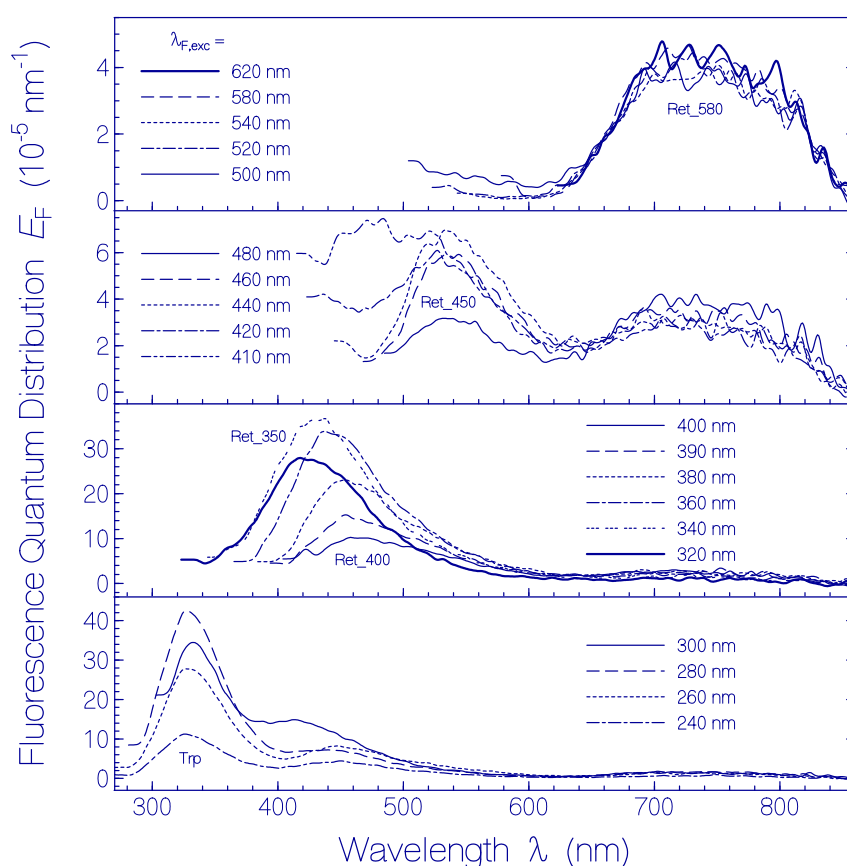
Only a slight increase of light scattering was found after 80 days of storage by comparing the attenuation coefficient spectra measured before and after sample centrifugation (for 20 min at 4400 rpm). The slow spectral changes indicate the high thermal stability of QuasAr1 at 2.5 °C.

Fluorescence emission quantum distributions  $E_F(\lambda)$  of QuasAr1 after 80 days of sample storage in the dark at 2.5 °C are shown in Figure 6. Fluorescence excitation in the wavelength range from  $\lambda_{F,\text{exc}} = 500$  nm to 620 nm (top sub-figure) resulted in the fluorescence emission band of Ret\_580 with fluorescence maximum around 740 nm. Sample excitation in the wavelength range from  $\lambda_{F,\text{exc}} = 420$  nm to 480 nm (second top sub-figure) revealed a second fluorescence emission band with emission maximum around 540 nm. It is attributed to a PRSB isomer named Ret\_450. In the second lowest sub-figure fluorescence emission spectra in the excitation wavelength region from  $\lambda_{F,\text{exc}} = 320$  nm to 400 nm are displayed. A weak fluorescence band peaking around 470 nm is resolved for fluorescence excitation around  $\lambda_{F,\text{exc}} \approx 400$  nm (RSB, Ret\_400). A stronger fluorescence band peaking around 440 nm is observed for fluorescence excitation around  $\lambda_{F,\text{exc}} \approx 350$  nm (RSB, Ret\_350). A short-wavelength fluorescence band with maximum around 330 nm, in the bottom sub-figure of Figure 6, belongs to the apoprotein Trp emission (absorption band maximum around  $\lambda_{F,\text{exc}} \approx 280$  nm). Fluorescence excitation in the wavelength range from  $\lambda_{F,\text{exc}} = 240$  nm to 280 nm additionally caused fluorescence emission around 450 nm. This indicates excitation energy transfer from apoprotein absorbing species Tyr and Trp to Ret\_350.

The excitation wavelength dependence of the total fluorescence quantum yield  $\phi_F$  of QuasAr1 in pH 8 Tris buffer after 80 days of storage at 2.5 °C in the dark is displayed by the line-connected circle curve in Figure 3. The spectral changes of  $\phi_F(\lambda_{F,\text{exc}})$  due to sample storage are seen easily by comparison with the  $\phi_F(\lambda_{F,\text{exc}})$  curve a fresh thawed QuasAr1 sample (dashed-line connected triangle curve in Figure 3). For  $\lambda_{F,\text{exc}} < 500$  nm the fluorescence quantum yield is increased by the thermally formed retinal isomers named Ret\_450, Ret\_400, and Ret\_350 and by the thermally induced apoprotein restructuring with fluorescence emission of Trp ( $\lambda_{F,\text{exc}}$  around 280 nm).

Normalized fluorescence excitation spectra of QuasAr1 in pH 8 Tris buffer stored in the dark at 2.5 °C for 80 days are presented in section S5 of the Supplementary Materials (Figure S5). They confirm the thermal formation of retinal isomers with increased fluorescence efficiency compared to Ret\_580 and the Trp fluorescence emission.





**Figure 6.** Fluorescence emission quantum distributions  $E_F(\lambda)$  of QuasAr1 in pH 8 Tris buffer stored at 2.5 °C for a duration of 80 days. The fluorescence excitation wavelengths  $\lambda_{F,exc}$  are listed in the legends.

#### 2.4. Temporal Development of QuasAr1 at Room Temperature

The thermal stability of QuasAr1 in pH 8 Tris buffer at room temperature ( $\vartheta = 21\text{--}25$  °C) in the dark was studied by carrying out transmission spectra measurements over a duration of 101 days and by measuring fluorescence emission and fluorescence excitation spectra after 50 days and at the end of the storage time.

The measured attenuation coefficient spectra  $\alpha(\lambda)$  are presented in Figure S6 of the Supplementary Materials (Section S6). In Figure 7a the temporal development of the absorption coefficient spectra  $\alpha_a(\lambda)$  is shown. The curves were derived from Figure S6 by removing the scattering contributions  $\alpha_s(\lambda)$  according to  $\alpha_a(\lambda) = \alpha(\lambda) - \alpha_s(\lambda)$  with  $\alpha_s(\lambda) = \alpha_s(\lambda_0) \times (\lambda_0/\lambda)^\gamma$  whereby  $\lambda_0$  was set to 800 nm and  $\gamma$  was adjusted in the transparency region (see below Section 4.2). The storage times are listed in the legend. The wavelength positions of maximum absorption of the originally present species (Ret\_580 and Trp) and the formed species (Ret\_530, Ret\_500, Ret\_450, Ret\_400, Ret\_350) are indicated.

In the main part of Figure 7a it is seen that the absorption decreased around 580 nm (PRSB, Ret\_580), and new absorption built-up and decreased around 500 nm (PRSB, Ret\_500). The absorption increased with storage time around 400 nm (RSB, Ret\_400) and around 350 nm (RSB, Ret\_350). The temporal increase of absorption below 320 nm is attributed to apoprotein restructuring with Trp enlarged absorption oscillator strength.

The inset of Figure 7a shows the dependence of the absorption coefficient  $\alpha_a(580\text{ nm})$  versus storage time  $t_{storage}$  (circles are experimental data). The decrease of the absorption coefficient with storage time is fitted by a two-component single exponential decay function according to:

$$\alpha_{a,580\text{ nm}}(t_{storage}) = \alpha_{a,580\text{ nm}}(0) \left[ \kappa_{Ret_{580,I}} \exp(-t_{storage}/\tau_{Ret_{580,I}}) + \kappa_{Ret_{580,II}} \exp(-t_{storage}/\tau_{Ret_{580,II}}) \right] \quad (1)$$

In Equation (1)  $\alpha_{a,580\text{ nm}}(0)$  is the total initial absorption coefficient at  $t_{\text{storage}} = 0$ .  $\kappa_{\text{Ret}_{580\text{I}}}$  is the fraction of Ret\_580 with fast absorption decay time constant  $\tau_{\text{Ret}_{580\text{I}}}$ . This component is named Ret\_580<sub>I</sub>.  $\kappa_{\text{Ret}_{580\text{II}}} = 1 - \kappa_{\text{Ret}_{580\text{I}}}$  is the fraction of Ret\_580 with slow absorption decay time constant  $\tau_{\text{Ret}_{580\text{II}}}$ . This component is named Ret\_580<sub>II</sub>. The fit parameters are  $\alpha_{a,580\text{ nm}}(0) = 2.193\text{ cm}^{-1}$ ,  $\kappa_{\text{Ret}_{580\text{I}}} = 0.41$ ,  $\tau_{\text{Ret}_{580\text{I}}} = 3.8\text{ d}$ ,  $\kappa_{\text{Ret}_{580\text{II}}} = 0.59$ , and  $\tau_{\text{Ret}_{580\text{II}}} = 120\text{ d}$ .

In Figure 7b the temporal development of absorption coefficient spectra of new formed species (Ret\_640, Ret\_530, Ret\_500, Ret\_450, Ret\_400, Ret\_350) are displayed. In the main part of Figure 7b the Ret\_580 contribution  $\alpha_{a,\text{Ret}_{580}}(\lambda, t_{\text{storage}})$  and the original residual retinal contributions  $\alpha_{a,\text{residual retinals}}(\lambda)$  are subtracted from  $\alpha_a(\lambda, t_{\text{storage}})$  of Figure 7a, i.e.:

$$\Delta\alpha_a(\lambda, t_{\text{storage}}) = \alpha_a(\lambda, t_{\text{storage}}) - \alpha_{a,\text{Ret}_{580}}(\lambda, t_{\text{storage}}) - \alpha_{a,\text{residual retinals}}(\lambda, 0) \quad (2)$$

is displayed. The curves show i) formation of a weak absorption band around 640 nm (formation of PRSB isomer Ret\_640), ii) build-up and decrease of a broad absorption band around 500 nm (formation of PRSB Ret\_500), iii) build-up of an absorption band around 400 nm (formation of RSB isomer Ret\_400), iv) build-up of an absorption band around 350 nm (formation of RSB isomer Ret\_350), and v) build-up of long-wavelength apoprotein absorption in the range  $< 340\text{ nm}$ . The Ret\_500 absorption band changed its shape for long-time sample storage  $t_{\text{storage}} \geq 50\text{ d}$ . Shoulders are seen around  $\lambda \approx 530\text{ nm}$  (Ret\_530) and around  $\lambda \approx 450\text{ nm}$  (Ret\_450). They may be due to new formed retinal isomer forms or due to Ret\_500 isomer position shift due to apoprotein adjacent structure changes. The minor part of the PRSB isomer Ret\_580 (fraction  $\kappa_{\text{Ret}_{580\text{I}}}$ , named Ret\_580<sub>I</sub>, likely a *cis* isomer) is converted dominantly to the short wavelength absorbing PRSB Ret\_500 (likely a *trans* isomer), and the dominant part of the PRSB isomer Ret\_580 (fraction  $\kappa_{\text{Ret}_{580\text{II}}}$ , named Ret\_580<sub>II</sub>, likely a *trans* isomer) is converted to the long-wavelength absorbing PRSB isomer Ret\_640 (likely a *cis* isomer). Ret\_500 is thought to deprotonate to Ret\_400 (likely a RSB *trans* isomer), and Ret\_640 is thought to deprotonate to Ret\_350 (likely a RSB *cis* isomer).

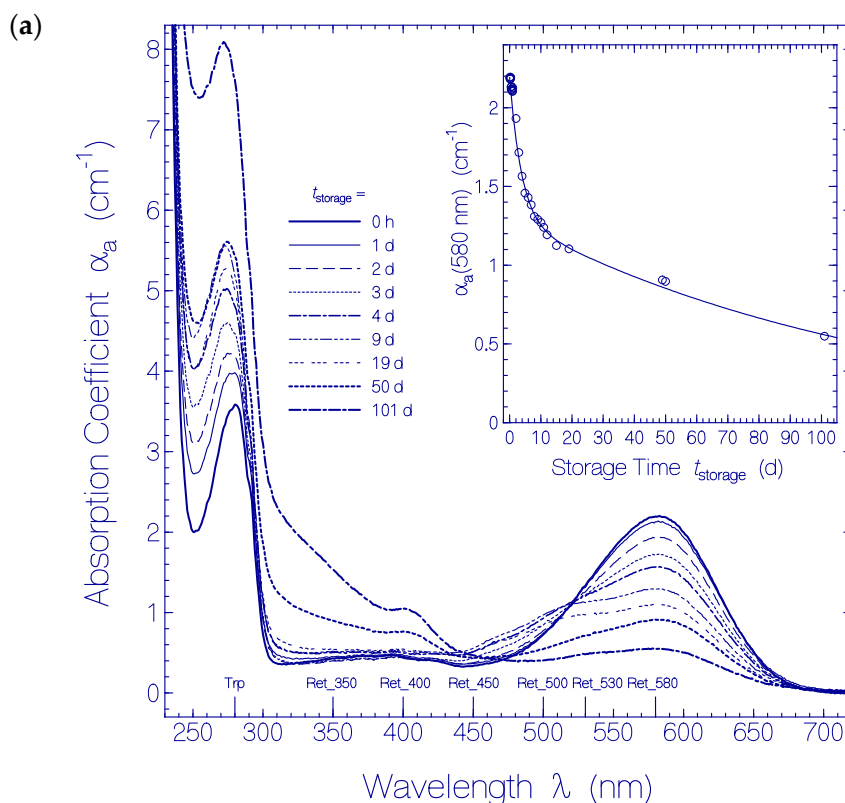
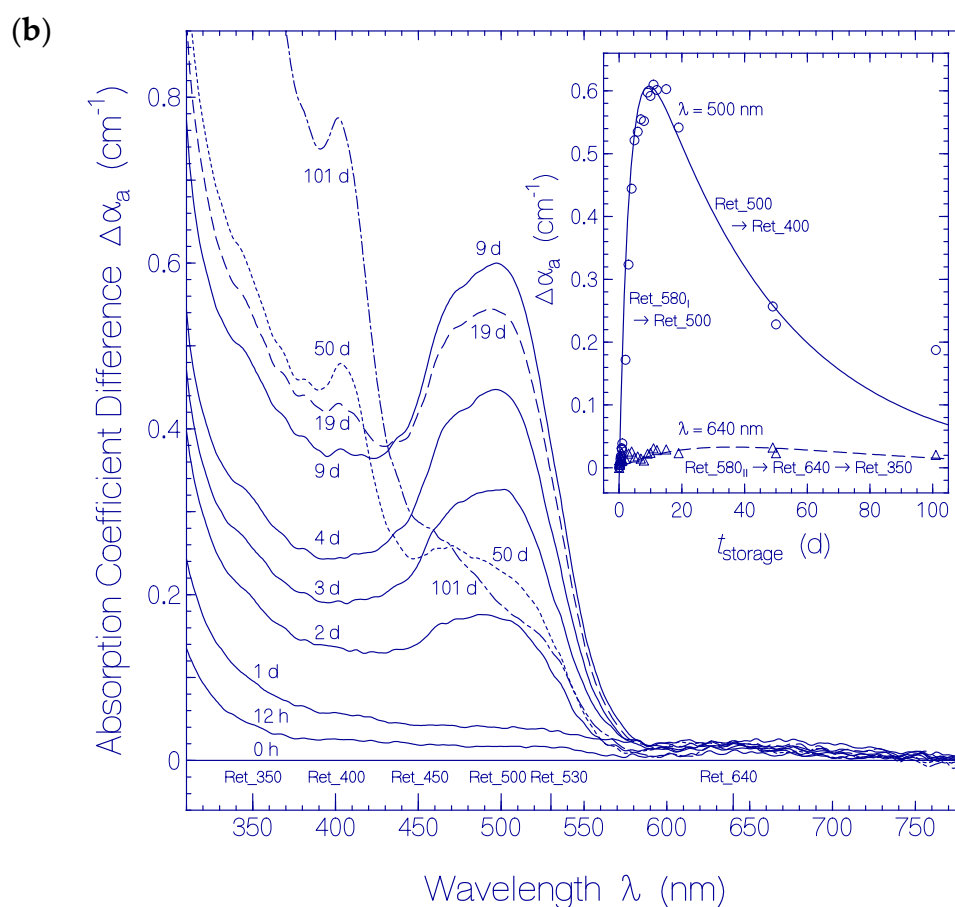


Figure 7. Cont.



**Figure 7.** (a) Temporal development of absorption coefficient spectra  $\alpha_a(\lambda)$  of QuasAr1 in pH 8 Tris buffer stored in the dark at room temperature ( $\vartheta = 21\text{--}25\text{ }^\circ\text{C}$ ). The storage times are listed in the legend ( $t_{\text{storage}} = 0\text{ h}$  refers to absorption coefficient spectrum measurement immediately after sample thawing). The inset shows the temporal development of  $\alpha_a(580\text{ nm})$  where the circles are data points and the solid curve is a two-component single exponential fit according to  $\alpha_{a,580\text{ nm}}(t_{\text{storage}}) = \alpha_{a,580\text{ nm}}(0) [\kappa_{\text{Ret}_{580\text{I}}} \exp(-t_{\text{storage}}/\tau_{\text{Ret}_{580\text{I}}}) + \kappa_{\text{Ret}_{580\text{II}}} \exp(-t_{\text{storage}}/\tau_{\text{Ret}_{580\text{II}}})]$  with  $\alpha_{a,580\text{ nm}}(0) = 2.193\text{ cm}^{-1}$ ,  $\kappa_{\text{Ret}_{580\text{I}}} = 0.41$ ,  $\tau_{\text{Ret}_{580\text{I}}} = 3.8\text{ d}$ ,  $\kappa_{\text{Ret}_{580\text{II}}} = 0.59$ , and  $\tau_{\text{Ret}_{580\text{II}}} = 120\text{ d}$ . (b) Temporal development of the corresponding difference absorption coefficient spectra  $\Delta\alpha_a(\lambda, t_{\text{storage}}) = \alpha_a(\lambda, t_{\text{storage}}) - \alpha_{a,\text{Ret}_{580}}(\lambda, t_{\text{storage}}) - \alpha_{a,\text{residual retinals}}(\lambda, 0)$ . The inset shows  $\Delta\alpha_a$  at  $\lambda = 500\text{ nm}$  and  $\lambda = 640\text{ nm}$  versus storage time  $t_{\text{storage}}$ . The  $\Delta\alpha_a$  data at  $\lambda = 500\text{ nm}$  are fitted by  $\Delta\alpha_{a,500\text{ nm}}(t_{\text{storage}}) = \Delta\alpha_{a,500\text{ nm,max}} [1 - \exp(-t_{\text{storage}}/\tau_{\text{Ret}_{580\text{I}}})] \exp(-t_{\text{storage}}/\tau_{\text{PT,I}})$  with  $\Delta\alpha_{a,500\text{ nm,max}} = 0.83\text{ cm}^{-1}$ ,  $\tau_{\text{Ret}_{580\text{I}}} = 3.8\text{ d}$ , and  $\tau_{\text{PT,I}} = 42\text{ d}$ . The  $\Delta\alpha_a$  data at  $\lambda = 640\text{ nm}$  are fitted by  $\Delta\alpha_{a,640\text{ nm}}(t_{\text{storage}}) = \Delta\alpha_{a,640\text{ nm,max}} [1 - \exp(-t_{\text{storage}}/\tau_{\text{Ret}_{580\text{II}}})] \exp(-t_{\text{storage}}/\tau_{\text{PT,II}})$  with  $\Delta\alpha_{a,640\text{ nm,max}} = 0.3\text{ cm}^{-1}$ ,  $\tau_{\text{Ret}_{580\text{II}}} = 120\text{ d}$ , and  $\tau_{\text{PT,II}} = 42\text{ d}$ .

The inset in Figure 7b displays the temporal development of  $\Delta\alpha_a(500\text{ nm})$  (circles, initial build-up of Ret\_500 due to conversion of Ret\_580<sub>I</sub> to Ret\_500 and subsequent decrease of Ret\_500 due to conversion to Ret\_400) and the temporal development of  $\Delta\alpha_a(640\text{ nm})$  (triangles, weak build-up of Ret\_640 due to conversion of Ret\_580<sub>II</sub> to Ret\_640 and concurrent conversion of Ret\_640 to Ret\_350). The temporal development of  $\Delta\alpha_a(500\text{ nm})$  is fitted by:

$$\Delta\alpha_{a,500\text{ nm}}(t_{\text{storage}}) = \Delta\alpha_{a,500\text{ nm,max}} [1 - \exp(-t_{\text{storage}}/\tau_{\text{Ret}_{580\text{I}}})] \exp(-t_{\text{storage}}/\tau_{\text{PT,I}}) \quad (3)$$

$\Delta\alpha_{a,500\text{ nm,max}}$  is the expected maximum  $\Delta\alpha_a(500\text{ nm})$  for  $t_{\text{storage}} \rightarrow \infty$  in the absence of deprotonation ( $\tau_{\text{PT,I}} \rightarrow \infty$ ).  $\tau_{\text{Ret}_{580\text{I}}}$  is the decay time constant of Ret\_580<sub>I</sub>.  $\tau_{\text{PT,I}}$  is the time constant of Ret\_500

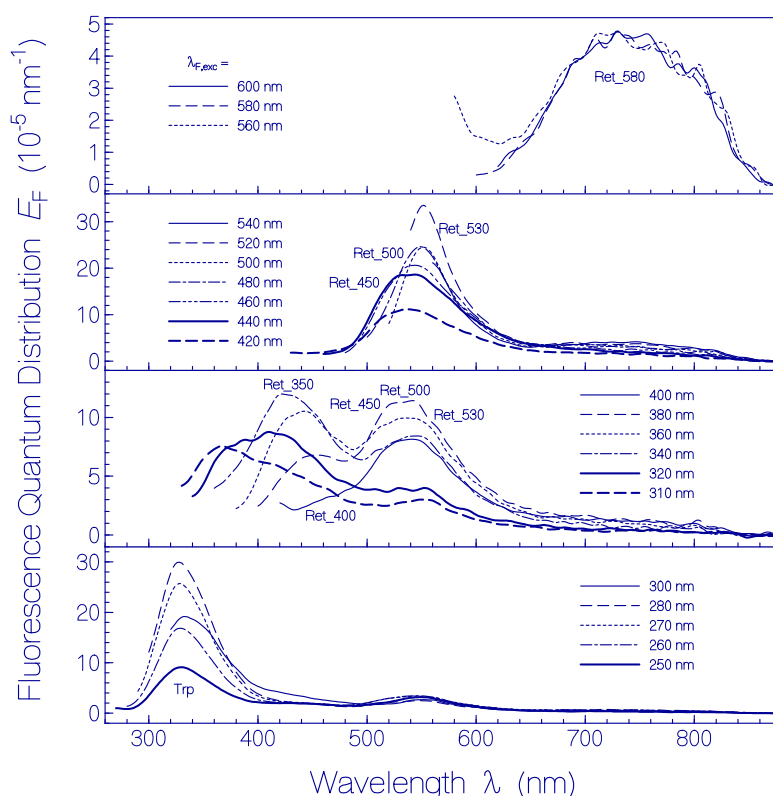
deprotonation. The fit parameters are  $\Delta\alpha_{a,500\text{ nm,max}} = 0.83\text{ cm}^{-1}$ ,  $\tau_{\text{Ret}_{580\text{I}}} = 3.8\text{ d}$ , and  $\tau_{\text{PT,II}} = 42\text{ d}$ . ( $\Delta\alpha_{a,500\text{ nm}}$  at  $t_{\text{storage}} = 101\text{ d}$  is larger than the fit value since short-wavelength absorption bands extend to  $\lambda = 500\text{ nm}$  and  $\Delta\alpha_{a,500\text{ nm}}(101\text{ d})$  is not only due to  $\text{Ret}_{500}$  absorption).

The temporal development of  $\Delta\alpha_a(640\text{ nm})$  is fitted by:

$$\Delta\alpha_{a,640\text{ nm}}(t_{\text{storage}}) = \Delta\alpha_{a,640\text{ nm,max}} \left[ 1 - \exp(-t_{\text{storage}}/\tau_{\text{Ret}_{580\text{II}}}) \right] \exp(-t_{\text{storage}}/\tau_{\text{PT,II}}) \quad (4)$$

$\Delta\alpha_{a,640\text{ nm,max}}$  is the expected maximum  $\Delta\alpha_a(640\text{ nm})$  for  $t_{\text{storage}} \rightarrow \infty$  in the absence of deprotonation ( $\tau_{\text{PT,II}} \rightarrow \infty$ ).  $\tau_{\text{Ret}_{580\text{II}}}$  is the decay time constant of  $\text{Ret}_{580\text{II}}$ .  $\tau_{\text{PT,II}}$  is the time constant of  $\text{Ret}_{640}$  deprotonation. The fit parameters are  $\Delta\alpha_{a,640\text{ nm,max}} = 0.3\text{ cm}^{-1}$ ,  $\tau_{\text{Ret}_{580\text{II}}} = 120\text{ d}$ , and  $\tau_{\text{PT,II}} = 42\text{ d}$ .

Fluorescence emission quantum distributions  $E_F(\lambda)$  of QuasAr1 after 50 days of sample storage in the dark at room temperature ( $\vartheta = 21\text{--}25\text{ }^\circ\text{C}$ ) are shown in Figure 8. Fluorescence excitation in the wavelength range of  $\lambda_{\text{F,exc}} \geq 560\text{ nm}$  (top sub-figure) resulted in the fluorescence emission band of  $\text{Ret}_{580}$  with fluorescence maximum around  $740\text{ nm}$ . Sample excitation in the region from  $\lambda_{\text{F,exc}} = 540\text{ nm}$  to  $\lambda_{\text{F,exc}} = 420\text{ nm}$  (second top sub-figure) resulted in peak fluorescence emission in the range from  $550\text{ nm}$  to  $530\text{ nm}$ . This emission is dominantly attributed to the formed protonated retinal Schiff base isomers  $\text{Ret}_{530}$ ,  $\text{Ret}_{500}$ , and  $\text{Ret}_{450}$ . In the second lowest sub-figure fluorescence emission spectra are resolved resulting from the deprotonated retinal Schiff base isomers  $\text{Ret}_{400}$  ( $\lambda_{\text{F,exc}} \approx 400\text{ nm}$ ,  $\lambda_{\text{F,max}} \approx 470\text{ nm}$ , weak fluorescence emission) and  $\text{Ret}_{350}$  ( $\lambda_{\text{F,exc}} \approx 350\text{ nm}$ ,  $\lambda_{\text{F,max}} \approx 430\text{ nm}$ ). In the bottom sub-figure the fluorescence emission peaking around  $\lambda_{\text{F,max}} \approx 330\text{ nm}$  is due to the apoprotein Trp emission (either directly excited or populated by excitation transfer from photo-excited Tyr to Trp).



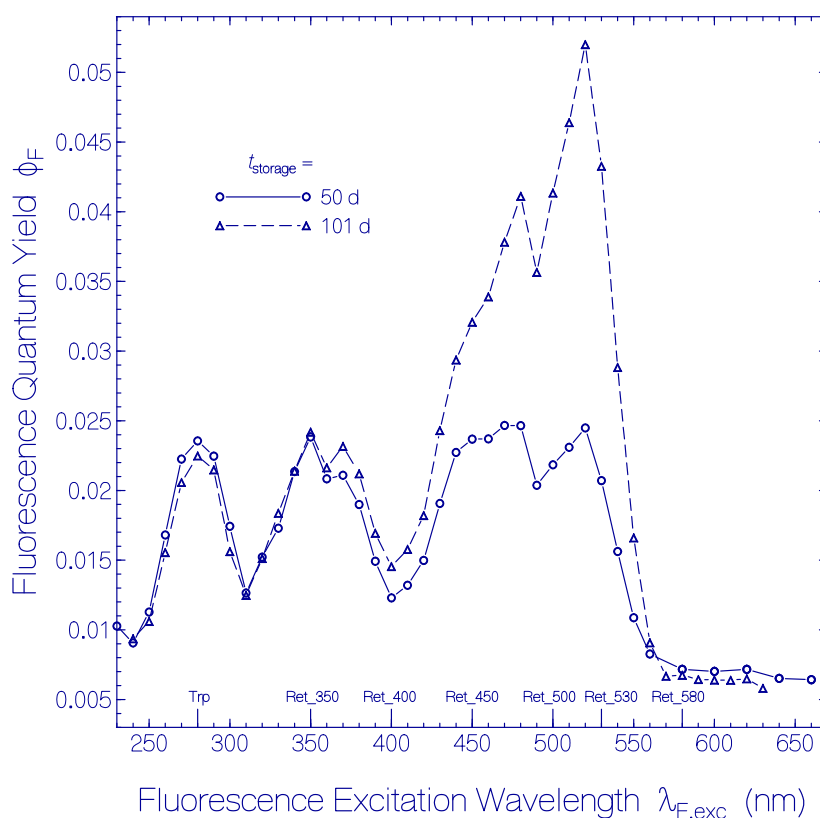
**Figure 8.** Fluorescence emission quantum distributions  $E_F(\lambda)$  of QuasAr1 in pH 8 Tris buffer stored at room temperature ( $\vartheta = 21\text{--}25\text{ }^\circ\text{C}$ ) for a duration of 50 days. The fluorescence excitation wavelengths  $\lambda_{\text{F,exc}}$  are listed in the legends.

In Figure S7 of the Supplementary Materials (Section S7) the fluorescence emission quantum distributions of QuasAr1 after 101 days of storage in the dark at room temperature are shown. The reduced presence of weakly fluorescing Ret\_580 increased the overall fluorescence quantum yield in the wavelength region from 420 nm to 540 nm (stronger fluorescent Ret\_530 and Ret\_450).

The excitation wavelength dependence of the fluorescence quantum yield  $\phi_F$  of QuasAr1 in pH 8 Tris buffer after 50 days and after 101 days of storage in the dark at room temperature is displayed in Figure 9.  $\phi_F$  is the ratio of the total amount of emitted fluorescence photons to the total amount of absorbed photons from the various absorbing species  $i$  in QuasAr1 at the selected excitation wavelength  $\lambda_{F,exc}$ . This means:

$$\phi_F(\lambda_{F,exc}) = \sum_i \frac{\alpha_{a,i}(\lambda_{F,exc})}{\alpha_a(\lambda_{F,exc})} \phi_{F,i}(\lambda_{F,exc}) \quad (5)$$

where  $i$  runs over the species absorbing at  $\lambda_{F,exc}$  with the absorption coefficients  $\alpha_{a,i}(\lambda_{F,exc})$ ,  $\phi_{F,i}(\lambda_{F,exc})$  is the fluorescence quantum yield of component  $i$ , and  $\alpha_a(\lambda_{F,exc}) = \sum_i \alpha_{a,i}(\lambda_{F,exc})$  is the total absorption coefficient at  $\lambda_{F,exc}$ . The wavelength positions of the absorption band maxima of the present components, protonated retinal Schiff bases Ret\_580, Ret\_530, Ret\_500, Ret\_450, deprotonated retinal Schiff bases Ret\_400 and Ret\_350, and the apoprotein contribution Trp are indicated at the bottom of Figure 9. The fluorescence quantum yield contributions of Ret\_500 and Ret\_400 are not well resolved. The fluorescence quantum yield of Ret\_580 of  $\phi_F \approx 0.007$  is the same after 50 days and 101 days of storage as of the fresh sample immediately after thawing. Ret\_530, Ret\_450, and Ret\_350 are a factor of 5 to 10 stronger fluorescent than Ret\_580. The fluorescence quantum yield of the apoprotein  $\phi_{F,Trp}$  is reduced by apoprotein excitation energy transfer to the retinal isomers.



**Figure 9.** Dependence of the total fluorescence quantum yield  $\phi_F$  on fluorescence excitation wavelength  $\lambda_{F,exc}$  for QuasAr1 in pH 8 Tris buffer stored in the dark at room temperature ( $\vartheta = 21\text{--}25\text{ }^\circ\text{C}$ ) for durations of 50 days and 101 days.

Normalized fluorescence excitation spectra of QuasAr1 in pH 8 Tris buffer stored in the dark at room temperature for 50 days (Figure S8) and 101 days (Figure S9) are presented in section S8 of the Supplementary Materials. They confirm the thermal formation of retinal isomers with increased fluorescence efficiency compared to Ret\_580 and the thermal apoprotein restructuring with increased absorption strength. The formation of small amounts of strongly fluorescent protonated retinal Schiff base isomers Ret\_530 and Ret\_450 by thermal activated ground-state isomerization of Ret\_580 are resolved in Figures S8 and S9.

### 3. Discussion

In Section 2 we reported spectroscopic investigation of QuasAr1 absorption and emission at pH 8 in Tris buffer. The samples were studied under different conditions: i) fresh thawed samples, ii) thermally aged samples at refrigerated temperature (2.5 °C) and room temperature (21–25 °C), and iii) heat-denatured samples. The measurements provided information on the thermal protein stability, the presence of different original apoprotein structures, the original retinal isomer composition, the thermal induced isomer conformation changes, the protonated retinal Schiff base isomers (PRSB) proton release to deprotonated retinal Schiff base isomers (RSB), and the thermal apoprotein restructuring of the originally present apoprotein structures showing up in UV spectral changes and absorption strength increase. The formation of new protonated retinal Schiff base isomers and their deprotonation to retinal Schiff base isomers occurred in parallel with the dynamic opsin apoprotein restructuring.

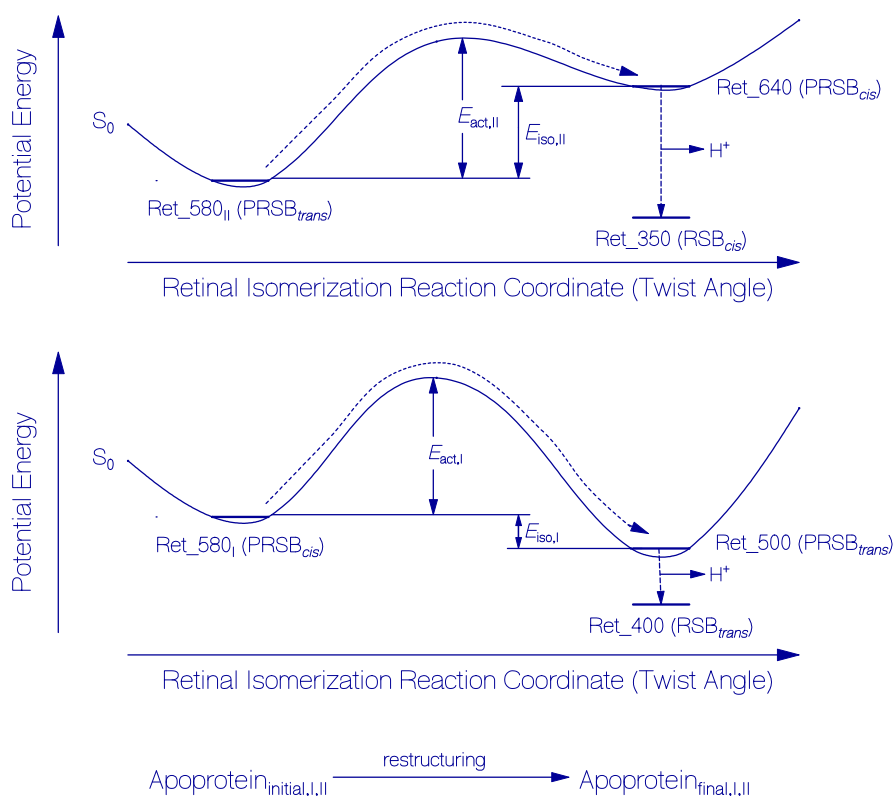
In the heterologous expression of QuasAr1 the retinal cofactor is covalently bound to the opsin protein via a lysine Schiff base. It is dominantly present in protonated form. About 86% of retinal was found to be present as protonated retinal Schiff base (PRSB) Ret\_580, and about 14% were found to be present mainly as neutral retinal Schiff base (RSB) isomers Ret\_400 and Ret\_350 and small amounts of other protonated retinal Schiff base isomers as Ret\_450 (see Figure 1 and section S4 of the Supplementary Materials).

At refrigerator temperature ( $\approx 2.5$  °C) over a period of 80 days only small conversion of Ret\_580 to Ret\_500, Ret\_450, Ret\_400 and Ret\_350 was observed. Some apoprotein restructuring showed up in increased UV absorption strength.

At room temperature ( $\approx 23$  °C) within the observation time of 101 days formation of new PRSB isomers (Ret\_640, Ret\_530, Ret\_500, Ret\_450) and significant deprotonation of the PRSB isomers to RSB isomers (Ret\_640 to Ret\_350, Ret\_500 to Ret\_400) occurred together with apoprotein restructuring showing up in increased UV absorption. The temporal two-component single exponential absorption decrease of Ret\_580 (Figure 7a) indicated its composition of two main isomer components Ret\_580<sub>I</sub> (likely a PRSB *cis* isomer in a specific QuasAr1 amino acid residue arrangement Apoprotein<sub>initial,I</sub>) and Ret\_580<sub>II</sub> (likely a PRSB *trans* isomer in another specific QuasAr1 amino acid residue arrangement Apoprotein<sub>initial,II</sub>). The temporal formation and decay of the absorption coefficient spectra of new formed species (Figure 7b) indicated i) a ground-state thermal activated isomerization of Ret\_580<sub>I</sub> to Ret\_500 (likely a PRSB *trans* isomer) and the subsequent deprotonation of Ret\_500 to Ret\_400 (likely a RSB *trans* isomer), and ii) a ground-state thermal activated isomerization of Ret\_580<sub>II</sub> to Ret\_640 (likely a PRSB *cis* isomer) and the concurrent deprotonation of Ret\_640 to Ret\_350 (likely a RSB *cis* isomer). QuasAr1 sample heating above 60 °C resulted in fast PRSB chromophore deprotonation to RSB. The thermal studies indicated energy barrier involved ground-state isomerizations, irreversible protein restructuring with irreversible protonated retinal Schiff base deprotonation and intrinsic apoprotein residue (mainly Trp) rearrangement with increased absorption oscillator strength.

The protonated retinal Schiff base ground-state isomerization, protonated retinal Schiff base deprotonation, and the apoprotein restructuring dynamics are illustrated in Figure 10. The top part illustrates the isomerization of the PRSB Ret\_580<sub>II</sub> isomer (likely all-*trans* isomer in a specific QuasAr1 protein conformation Apoprotein<sub>II</sub>) to the PRSB Ret\_640 isomer (likely 13-*cis* isomer in the same specific QuasAr1 protein conformation) and the concurrent proton release from Ret\_640 to the stable formation of Ret\_350 (likely a RSB 13-*cis* isomer). The middle part illustrates the isomerization of the PRSB

Ret<sub>580<sub>I</sub></sub> isomer (likely 13-*cis* isomer in a specific QuasAr1 protein conformation Apoprotein<sub>I</sub>) to the PRSB Ret<sub>500</sub> isomer (likely all-*trans* isomer in the same specific QuasAr1 protein conformation) and the subsequent proton release from Ret<sub>500</sub> to the stable formation of Ret<sub>400</sub> (likely a RSB all-*trans* isomer). The bottom part illustrates the concurrent occurring apoprotein restructuring supporting the ground-state protonated retinal Schiff base isomerizations and deprotonations.



**Figure 10.** Schematic reaction coordinate diagrams for thermal activated  $S_0$  ground-state protonated retinal Schiff base isomerizations and apoprotein restructuring assisted irreversible deprotonations to retinal Schiff base isomers. Top part: Isomerization of Ret<sub>580<sub>II</sub></sub> to Ret<sub>640</sub> and subsequent deprotonation to Ret<sub>350</sub>. Middle part: Isomerization of Ret<sub>580<sub>I</sub></sub> to Ret<sub>500</sub> and subsequent deprotonation to Ret<sub>400</sub>. Bottom part: Parallel occurring opsin restructuring of originally present apoprotein structures (Apoprotein<sub>initial,I</sub> and Apoprotein<sub>initial,II</sub>) to final apoprotein structures (Apoprotein<sub>final,I</sub> and Apoprotein<sub>final,II</sub>) of QuasAr1 acting on protonated retinal Schiff base isomerization and deprotonation.

The energetic level positions  $E_{iso,I}$  of Ret<sub>500</sub> and  $E_{iso,II}$  of Ret<sub>640</sub> may be estimated from the expected maximum absorption differences  $\Delta\alpha_{a,500\text{ nm,max}}$  and  $\Delta\alpha_{a,640\text{ nm,max}}$  of Equations (3) and (4). Assuming equal absorption cross-sections  $\sigma_{a,\text{Ret}_{580}}(580\text{ nm})$ ,  $\sigma_{a,\text{Ret}_{500}}(500\text{ nm})$ , and  $\sigma_{a,\text{Ret}_{640}}(640\text{ nm})$ , the fractions  $\chi_{\text{Ret}_{500}}$  and  $\chi_{\text{Ret}_{640}}$  of thermally populated Ret<sub>500</sub> and Ret<sub>640</sub> would be [54]:

$$\chi_{\text{Ret}_{500}} = \frac{\Delta\alpha_{a,500\text{ nm,max}}}{\alpha_{a,580\text{ nm}}(0)\kappa_{\text{Ret}_{580,I}}} \quad (6)$$

$$\chi_{\text{Ret}_{640}} = \frac{\Delta\alpha_{a,640\text{ nm,max}}}{\alpha_{a,580\text{ nm}}(0)\kappa_{\text{Ret}_{580,II}}} \quad (7)$$

The energy level positions  $E_{iso,I}$  and  $E_{iso,II}$  are obtained by application of the Boltzmann level position law [72]:

$$\chi_{\text{Ret}_{500}} = \frac{\exp(-E_{iso,I}/(k_B\vartheta))}{1 + \exp(-E_{iso,I}/(k_B\vartheta))} \quad (8)$$

and:

$$\chi_{\text{Ret}_640} = \frac{\exp(-E_{\text{iso},II}/(k_B\vartheta))}{1 + \exp(-E_{\text{iso},II}/(k_B\vartheta))} \quad (9)$$

where  $k_B$  is the Boltzmann constant and  $\vartheta$  is the temperature.

Solving Equations (8) and (9) for  $E_{\text{iso},I}$  and  $E_{\text{iso},II}$  gives:

$$E_{\text{iso},I} = -\ln\left(\frac{\chi_{\text{Ret}_500}}{1 - \chi_{\text{Ret}_500}}\right)k_B\vartheta \quad (10)$$

and:

$$E_{\text{iso},II} = -\ln\left(\frac{\chi_{\text{Ret}_640}}{1 - \chi_{\text{Ret}_640}}\right)k_B\vartheta \quad (11)$$

Insertion of parameters gives:  $\chi_{\text{Ret}_500} = 0.92$  ( $\Delta\alpha_{a,500 \text{ nm,max}} = 0.83 \text{ cm}^{-1}$ ,  $\alpha_{a,580 \text{ nm}}(0) = 2.193 \text{ cm}^{-1}$ ,  $\kappa_{\text{Ret}_580,I} = 0.41$ ),  $\chi_{\text{Ret}_640} = 0.23$  ( $\Delta\alpha_{a,500 \text{ nm,max}} = 0.3 \text{ cm}^{-1}$ ,  $\alpha_{a,580 \text{ nm}}(0) = 2.193 \text{ cm}^{-1}$ ,  $\kappa_{\text{Ret}_580,II} = 0.59$ ),  $E_{\text{iso},I} = -9.98 \times 10^{-21} \text{ J} = -500 \text{ cm}^{-1} \times hc_0$  ( $k_B = 1.38 \times 10^{-23} \text{ J K}^{-1}$ ,  $\vartheta = 296 \text{ K}$  is temperature,  $h$  is the Planck constant, and  $c_0$  is the speed of light in vacuum), and  $E_{\text{iso},II} = 4.94 \times 10^{-21} \text{ J} = 248 \text{ cm}^{-1} \times hc_0$ .

The time constants of  $\text{Ret}_{580,I}$  isomerization to  $\text{Ret}_{500}$ ,  $\tau_{\text{Ret}_{580,I}}$ , of  $\text{Ret}_{580,II}$  isomerization to  $\text{Ret}_{640}$ ,  $\tau_{\text{Ret}_{580,II}}$ , of  $\text{Ret}_{500}$  deprotonation to  $\text{Ret}_{400}$ ,  $\tau_{PT,I}$ , and of  $\text{Ret}_{640}$  deprotonation to  $\text{Ret}_{350}$ ,  $\tau_{PT,II}$ , were determined above (Equations (3) and (4)). They are related to the energy activation barriers  $E_{\text{act},I}$ ,  $E_{\text{act},II}$ ,  $E_{\text{act},PT,I}$ , and  $E_{\text{act},PT,II}$  by the Arrhenius relation [73] according to:

$$\tau_{\text{Ret}_{580,I}} = \tau_0 \exp\left(\frac{E_{\text{act},I}}{k_B\vartheta}\right) \quad (12)$$

$$\tau_{\text{Ret}_{580,II}} = \tau_0 \exp\left(\frac{E_{\text{act},II}}{k_B\vartheta}\right) \quad (13)$$

$$\tau_{PT,i} = \tau_0 \exp\left(\frac{E_{\text{act},PT,i}}{k_B\vartheta}\right), \quad i = I, II \quad (14)$$

where  $\tau_0 = h/(k_B\vartheta)$  is the attempt time constant of barrier crossing [59,74]. Solving Equations (12)–(14) for the activation energy barriers gives:

$$E_{\text{act},I} = \ln\left(\frac{\tau_{\text{Ret}_{580,I}}}{\tau_0}\right)k_B\vartheta \quad (15)$$

$$E_{\text{act},II} = \ln\left(\frac{\tau_{\text{Ret}_{580,II}}}{\tau_0}\right)k_B\vartheta \quad (16)$$

$$E_{\text{act},PT,i} = \ln\left(\frac{\tau_{PT,i}}{\tau_0}\right)k_B\vartheta, \quad i = I, II \quad (17)$$

Insertion of parameters ( $\vartheta = 296 \text{ K}$ ,  $\tau_0 \approx 1.6 \times 10^{-13} \text{ s}$ ,  $\tau_{\text{Ret}_{580,I}} = 3.8 \text{ d}$ ,  $\tau_{\text{Ret}_{580,II}} = 120 \text{ d}$ ,  $\tau_{PT,I} \approx \tau_{PT,II} \approx 42 \text{ d}$ ) leads to  $E_{\text{act},I} = 1.72 \times 10^{-19} \text{ J} = 8670 \text{ cm}^{-1} \times hc_0$ ,  $E_{\text{act},II} = 1.86 \times 10^{-19} \text{ J} = 9380 \text{ cm}^{-1} \times hc_0$ , and  $E_{\text{act},PT,I} \approx E_{\text{act},PT,II} \approx 1.82 \times 10^{-19} \text{ J} \approx 9160 \text{ cm}^{-1} \times hc_0$ .

The performed data analysis of fresh thawed and of heat treated QuasAr1 allowed to determine the absorption cross-section spectra of PRSB  $\text{Ret}_{580}$  (a composition of PRSB 13-*cis* isomer  $\text{Ret}_{580,I}$  and PRSB all-*trans* isomer  $\text{Ret}_{580,II}$  in two different protein adjacent amino acid arrangements Apoprotein<sub>initial,I</sub> and Apoprotein<sub>initial,II</sub>) and of the RSB  $\text{Ret}_{380}$  (likely unresolved composition of  $\text{Ret}_{350}$  and  $\text{Ret}_{400}$ ) which are shown in Figure S3. Knowledge of the absorption cross-section spectra



allowed the determination of the radiative lifetimes  $\tau_{rad}$  of the  $S_1$ – $S_0$  emission transitions using the Strickler–Berg formula according to [75–77]:

$$\tau_{rad} = \frac{n_A \bar{\lambda}_F^{-3}}{8\pi c_0 n_F^3 \bar{\sigma}} \quad (18)$$

where  $n_A$  and  $n_F$  are the average refractive indices of the aqueous buffer solution in the  $S_0$ – $S_1$  absorption band region and the  $S_1$ – $S_0$  emission band region, respectively, and  $c_0$  is the velocity of light in vacuum.  $\bar{\lambda}_F = \left( \int_{em} E_F(\lambda) \lambda^3 d\lambda / \int_{em} E_F(\lambda) d\lambda \right)^{1/3}$  is the average  $S_1$ – $S_0$  fluorescence emission wavelength, and  $\bar{\sigma}_a = \int_{abs} (\sigma_a(\lambda) / \lambda) d\lambda$  is the absorption cross-section strength of the  $S_0$ – $S_1$  absorption band. Using appropriate absorption cross-section data from Figure S3, fluorescence quantum distribution data from Figure 6 and refractive indices of water, we determine  $\tau_{rad}(\text{Ret}_{580}) = 9.32$  ns ( $n_F = 1.33$ ,  $n_A = 1.3328$ ,  $\bar{\lambda}_F = 745$  nm,  $\bar{\sigma}_a = 3.33 \times 10^{-17}$  cm<sup>2</sup>) and  $\tau_{rad}(\text{Ret}_{380}) \approx 3.74$  ns ( $n_F = 1.3366$ ,  $n_A = 1.3406$ ,  $\bar{\lambda}_F \approx 460$  nm,  $\bar{\sigma}_a = 1.94 \times 10^{-17}$  cm<sup>2</sup>).

Average Strickler–Berg based fluorescence lifetimes  $\tau_{F,SB}$  are obtained from the radiative lifetimes and the fluorescence quantum yields according to:

$$\tau_{F,SB} = \phi_F \tau_{rad} \quad (19)$$

The obtained values are  $\tau_{F,SB}(\text{Ret}_{580}) \approx 61.5$  ps ( $\phi_F \approx 0.0065$ ) and  $\tau_{F,SB}(\text{Ret}_{380}) \approx 150$  ps ( $\phi_F \approx 0.04$ ).

The fluorescence quantum yields and the fluorescence lifetimes of the protonated retinal Schiff base chromophore Ret<sub>580</sub> of fresh thawed QuasAr1 and of the thermally formed deprotonated retinal Schiff base Ret<sub>380</sub> are extraordinary large compared to the parent wild-type Archaeorhodopsin 3 ( $\phi_F(\text{Arch}) = 9 \times 10^{-4}$ ) [36,42]. The performed mutations on Arch to get QuasAr1 led to a slowing down of the excited-state isomerization dynamics via twisted internal conversion ( $S_1$ – $S_0$  conical intersection). They cause some restriction (barrier) along the reactive coordinate (twist angle) of photoisomerization. The slower relaxation along the  $S_1$  state potential energy surface towards the funnel position of  $S_1$  to  $S_0$  internal conversion leads to the broad-band fluorescence emission of increased efficiency and longer fluorescence lifetime ([51] and references therein). In Table S1 of the Supplementary Materials (section S9) absorption, fluorescence, and primary photoisomerization parameters of some rhodopsins are collected for comparison.

## 4. Materials and Methods

### 4.1. Sample Preparation

QuasAr1 gene was a gift from Adam E. Cohen (Addgene plasmid # 64135, [42]). *E. coli* optimized gene was cloned into pET21a(+) vector between the NdeI and SalI restriction sites with a C-terminal TEV protease cleavage site and a HIS<sub>6</sub> tag (ENLYFQSLVDLEHHHHHH).

The expression plasmid (pet21a+) carrying QuasAr1 was transformed into C41(DE3) *E. coli* cells. To induce the protein expression we used 0.5 mM isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG; Carl Roth GmbH, Karlsruhe, Germany) and the LB media was supplemented with 5  $\mu$ M all-trans retinal (ATR; Sigma-Aldrich, St. Louis, USA). The cells were incubated at 37 °C for 4 h and then harvested. The cells were disrupted using an EmulsiFlex-C3 Homogenizer (AVESTIN Inc., Ottawa, Canada). The membrane fraction was collected by ultracentrifugation (45,000 rpm) for 1 h at 4 °C (Type 45 Ti; Beckman Inc., Indianapolis, USA) and then resuspended in buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1.5% n-dodecyl- $\beta$ -D-maltoside (DDM, GLYCON Biochemicals GmbH, Luckenwalde, Germany), and 0.3% cholesteryl hemisuccinate (CHS, Sigma-Aldrich, St. Louis, USA) and stirred overnight for solubilization. The insoluble fraction was removed by ultracentrifugation (200,000  $\times$  g, 1 h at 4 °C). The QuasAr1 protein was purified by Ni-NTA affinity and using an ÄKTAexpress protein purification system (GE Healthcare Life Science,

Chicago, USA) configured with a HisTrap HP Ni-NTA column. The protein was collected in the final buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% DDM, 0.004% CHS, 0.1 mM PMSF, and 5% glycerol.

The expressed QuasAr1 protein in the final buffer was aliquoted to amounts of 30  $\mu$ L in Eppendorf tubes, shock-frozen, and stored at  $-80$  °C until thawing for experimental investigations.

#### 4.2. Spectroscopic Measurements

Transmission measurements,  $T(\lambda)$  ( $\lambda$  is wavelength), were carried out with a spectrophotometer (Cary 50, Varian Australia Pty Ltd, Mulgrave, Victoria, Australia). Attenuation coefficient spectra were calculated by the relation,  $\alpha(\lambda) = -\ln[T(\lambda)]/l$ , where  $l$  is the sample length. In the case of negligible protein light scattering the attenuation coefficient spectrum  $\alpha(\lambda)$  is equal to the absorption coefficient spectrum  $\alpha_a(\lambda)$ . Otherwise it comprises absorption ( $\alpha_a$ ) and scattering ( $\alpha_s$ ) contributions according to  $\alpha(\lambda) = \alpha_a(\lambda) + \alpha_s(\lambda)$ . The scattering coefficient spectrum is approximated by the empirical relation [78]  $\alpha_s(\lambda) = \alpha_s(\lambda_0)(\lambda_0/\lambda)^\gamma$  where the wavelength  $\lambda_0$  is selected in the transparency region and  $\gamma \leq 4$  is fitted to the experimental attenuation in the transparency region. Absorption coefficient spectra became available by subtracting the scattering contribution from the measured attenuation coefficient spectra.

The QuasAr1 melting was studied by stepwise sample heating up, then cooling down and thereby measuring the attenuation coefficient spectra development [58,59]. The apparent protein melting temperature  $\vartheta_m$  was derived from the onset of strong light attenuation in the transparency region of QuasAr1.

The thermal stability of QuasAr1 at room temperature (21–25 °C) and refrigerator temperature ( $2.5 \pm 0.5$  °C) was determined by storing QuasAr1 samples at these temperatures in the dark and measuring transmission spectra at certain time intervals.

Fluorescence spectroscopic measurements were carried out with a spectrofluorimeter (Cary Eclipse, Varian Australia Pty Ltd, Mulgrave, Victoria, Australia). Fluorescence emission quantum distributions  $E_F(\lambda)$  were determined from fluorescence emission spectra measurements at fixed excitation wavelengths [52,79,80]. The dye rhodamine 6G in methanol was used as reference standard for fluorescence quantum distribution calibration (fluorescence quantum yield  $\phi_{F,\text{ref}} = 0.93$  [81]). The fluorescence quantum yield  $\phi_F$  was calculated using the relation  $\phi_F = \int_{em} E_F(\lambda)d\lambda$  where the integration runs over the fluorescence emission wavelength region. Fluorescence excitation quantum distributions  $E_{ex}(\lambda)$  were recorded by scanning the fluorescence excitation wavelength over the absorption wavelength region at fixed fluorescence detection wavelengths [82]. Magic angle conditions were applied for the fluorescence recordings (vertical polarized excitation and orientation of the fluorescence detection polarizer at an angle of  $54.7^\circ$  to the vertical [83]). The spectra were corrected for the spectral sensitivity of the spectrometer and the photodetector.

## 5. Conclusions

The rhodopsin fluorescent voltage sensor QuasAr1 [42] was characterized by its absorption and emission spectroscopic behavior and its long-time thermal stability. At refrigerator temperature it may be used over a period of about 40 days without significant absorption and fluorescence spectroscopic changes. At room temperature it is possible to store the voltage sensor over about one day without severe retinal Schiff base deprotonation and opsin protein restructuring.

In the dark at room temperature the formation of new retinal isomers in the ground-state of QuasAr1 took place by thermal overcoming of energy barriers and by lowering the potential energy levels of the protonated Schiff base isomers (dominantly Ret\_500 and Ret\_640) and the deprotonated Schiff base isomers (dominantly Ret\_400 and Ret\_350) due to dynamic protein restructuring on a time scale of days. The isomerization dynamics of QuasAr1 retinals in the excited state (photoisomerization) occurs on a ten picosecond timescale due to a different barrier-involved  $S_1$  state potential energy surface structure.

**Supplementary Materials:** Supplementary Materials can be found at <http://www.mdpi.com/1422-0067/20/17/4086/s1>. References [84–103] are cited in the supplementary materials

**Author Contributions:** The study was initiated by A.S. and P.H. who expressed, purified and delivered the protein. A.P. carried out the absorption and emission spectroscopic measurements. The manuscript was written by A.P. and commented and improved by A.S. and P.H.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

Arch	Archaerhodopsin 3 from <i>Halorubrum sodomense</i>
GECI	Genetically encoded calcium indicator
GEVI	Genetically encoded voltage indicator
PRSB	Protonated retinal Schiff base
QuasAr	Quality superior to Arch
Ret_xxx	Retinal with absorption maximum approximately at xxx nm
RSB	Retinal Schiff base
Trp	Tryptophan
Tyr	Tyrosine
VSD	Voltage sensing domain

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