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Temperature Independence of Ultrafast Photoisomerization in Thermophilic Rhodopsin: Assessment versus Other Microbial Proton Pumps

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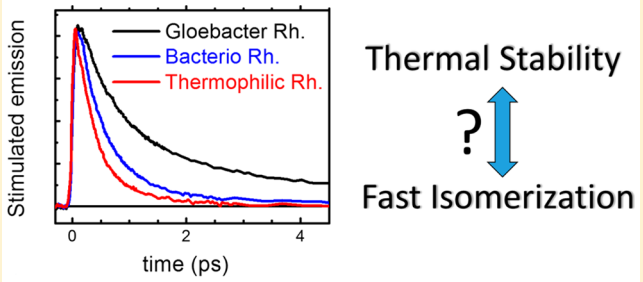
Supporting Information

ABSTRACT: Primary photochemical events in the unusually thermostable proton pumping rhodopsin of *Thermus thermophilus* bacterium (TR) are reported for the first time. Internal conversion in this protein is shown to be significantly faster than in bacteriorhodopsin (BR), making it the most rapidly isomerizing microbial proton pump known. Internal conversion (IC) dynamics of TR and BR were recorded from room temperature to the verge of thermal denaturation at 70 °C and found to be totally independent of temperature in this range. This included the well documented multiexponential nature of IC in BR, suggesting that assignment of this to ground state structural inhomogeneity needs revision. TR photodynamics were also compared with that of the phylogenetically more similar proton pump *Gloeobacter* rhodopsin (GR). Despite this similarity GR has poor thermal stability, and the excited state decays significantly more slowly and exhibits very prominent stretched exponential behavior. Coherent torsional wavepackets induced by impulsive photoexcitation of TR and GR show marked resemblance to each other in frequency and amplitude and differ strikingly from similar signatures in pump−probe data of BR and other microbial retinal proteins. Possible correlations between IC rates and thermal stability and the promise of using torsional coherence signatures for understanding chromophore protein binding in microbial retinal proteins are discussed.



INTRODUCTION

The ultrafast nature and high quantum efficiency of photoisomerization in retinal proteins (RPs) have intrigued photobiologists for decades.[1−9] While photochemistry has only been studied extensively in a single visual pigment, the bovine rhodopsin,[9−14] similarly investigated microbial ion pumps and sensory rhodopsins now number more than ten.[8,15−22] Aside from upholding the singularly fast internal conversion (IC) in bovine rhodopsin (Rh), study of the microbial retinal proteins (MRP or type I) clearly demonstrates the decisive control exerted by the opsin environment on the photochemistry of the embedded chromophore, including directing activity to a single C═C bond and shifting the rate of isomerization up to 10-fold. It has also systematically disproved suggested correlations of isomerization rates with λ_{max} of absorption (opsin shifts),[19,23] photoisomerization efficiencies, and other protein specific parameters.[24]

Meanwhile, modern genomic tools have facilitated discovery of enticing new members of the MRP family.[25,26] In 2012, a gene coding for a retinal proton pump was discovered in the bacterium *Thermus thermophilus* JL-18, isolated from hot springs in the United States great basin.[27] Coined TR, this protein is remarkably stable at temperatures in excess of 80 °C, even in a detergent stabilized state outside its native membrane.[28,29] This enhanced thermal stability is unique among known MRPs, which denature or lose their chromophore absorption quite rapidly at this temperature.[30−32]

In view of the ongoing conundrum surrounding the factors that govern the broad range of IC rates in MRPs, investigating IC in TR is of particular interest. The evolutionary adaptation of this protein to function at high temperatures must involve structural reinforcement, which might influence IC rates as a side-effect. Furthermore, the effects of such structural "hardening" should become more apparent as the temperature is raised.

The enhanced thermal stability of TR has been ascribed to $\pi-\pi$ interactions between aromatic residues in the extracellular domain, as well as interhelical hydrogen bonds in the membranal domain, which enhance the proteins resistance to thermal disruptions.[33] Regardless of the atomistic details, the thermal stabilization of TR, allowing it to function in harsh thermal environments without denaturation, must involve

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increased forces maintaining the native structure and a more rigid binding pocket for the chromophore. These forces should contribute to steepen the potentials related to the *trans* to *cis* reaction and possibly hasten or at least influence the transition time scale.

The structural hardening might also bear on the significance of the extreme rate of primary events in bovine rhodopsin.[13,14] Numerous studies cite this briefness as a key motivation for investigating rhodopsin photochemistry. However, it is hard to see how this enhanced rate impacts the millisecond time domain process of visual transduction. As a dim-light photoreceptor, rhodopsin has been thermally silenced to reduce the rate of ground state isomerization per pigment in the living eye to once in $\sim 10^3$ years![34−36] This silencing must involve enhancing barriers to thermal isomerization, leading again to increased steepness of potential energy variations along the torsional motions that facilitate *trans*−*cis* isomerization. Thus, despite the significant differences in the evolutionary pressure leading to these two adaptations, thermal silencing in Rh and stabilization in TR could hasten photochemical rates in both.

Among other MRP proton pumps, the archetypal bacteriorhodopsin (BR) is itself relatively thermostable when embedded in its native purple membrane.[31,37] Unlike TR, which is nearly unaffected when heated to 80 °C, BR is known to undergo spectral changes upon heating. These changes are reversible up to 65 °C. An irreversible change is observed above the melting temperature of ∼90 °C.[38] During this elevation in temperature, the absorption spectrum of bacteriorhodopsin is blue-shifted, broadened, and reduced in peak absorbance. These changes have been tentatively assigned to altered tertiary structure of the protein, but details of these changes have yet to be determined.[39,40]

In terms of amino acid sequence, TR is much closer to the bacterial proton pumps xanthorhodopsin (XR) and *Gloeobacter* rhodopsin (GR) (54%/83% identity/similarity) than it is to archaeal BR.[28] Strangely this sequence similarity does not encode the thermal stability, and GR is not only less thermostable than TR but even less so than BR. Unlike BR, both dark and light adaptation lead to all-*trans* retinal resting state in TR, and the room temperature photocycle takes ∼280 ms. This cycle is shortened by more than an order of magnitude at 50 °C, so that under this extremophile's ambient conditions the duration of the photocycle is comparable to that in BR.[28,29,41]

In the present study, primary events in TR are monitored for the first time at various temperatures and compared to similar events in BR and GR. The aim of this investigation is to characterize the course of photoisomerization in TR for the first time and in particular to find out whether the course of structural stabilization of this protein against thermal fluctuations affects those dynamics. Results show that IC in TR is much faster than that in GR and even faster than that in BR, the current record holder among microbial proton pumps. In this limited context, the rate of IC is correlated with thermal stability of the pigments. Photochemical dynamics in TR and in BR are unaffected by variation of temperature from 20 to 70 °C. This includes the characteristic low frequency modes associated with skeletal motions, which are similar in TR and GR and quite different in frequency from those recorded in BR.

EXPERIMENTAL SECTION

TR was prepared following a slightly modified procedure reported in ref 28. In brief, codon optimized TR was expressed in Luria−Bertani (LB) medium in *Escherichia coli* BL21(DE3) cells. *E. coli* transformants were grown to A600 = 1.30 in the presence of ampicillin (50 μg/mL) at 37 °C. Then, the cells were induced with 0.1% L(+)-arabinose and 10 μM all-*trans* retinal for 4 h. Pink-colored cells were harvested by centrifugation at 4 °C, followed by resuspension with buffer S (50 mM MeS, 300 mM NaCl, 5 mM imidazole, 5 mM $MgCl_2$; pH 6) containing 1% *n*-dodecyl-β-D-maltoside (DDM) and lysed with lysozyme (0.1 mg/mL) in the presence of DNase and protease inhibitor. The mixture was stirred overnight at room temperature. The extracted protein was collected as supernatant after centrifugation of the stirred solution at 18 000 rpm and 4 °C for 25 min. The protein was purified by using a Ni^{2+}-NTA histidine-tagged agarose column. The histidine-tagged protein was washed with buffer W (50 mM MeS, 300 mM NaCl, 50 mM imidazole, 0.06% DDM; pH 6) and eluted with buffer E (0.06% DDM, 50 mM Tris-HCl, 300 mM NaCl, 50 mM HCl, 150 mM imidazole; pH 7.5). Eluted protein was washed and concentrated using 0.02% DDM solution that contains 300 mM NaCl using Amicon Ultra centrifugal filter devices. BR was prepared using the procedure reported elsewhere.[42] GR was expressed in *E. coli*, and the cells were induced with 0.2% L(+)-arabinose and 10 μM all-*trans* retinal. GR was purified following similar procedure as TR, and the eluted protein was washed and concentrated using 0.02% DDM solution. The final solutions of BR and GR contained 300 mM NaCl.

Steady state absorption spectra were recorded on an Ocean optics HR2000 spectrophotometer. The sample reservoir and the flow-cell were heated with a circulating water bath. The experiments were performed at 22, 50, and 70 °C. The same heating arrangement was used for time-resolved experiments as well. GR was not tested at elevated temperatures since it has been reported to decompose rapidly above room temperature when solubilized in detergent. As shown by Neebe et al., the absorption spectrum of BR undergoes blue shifting by ∼40 nm accompanied by broadening upon heating (Figure S1).[38] The absorption changes are reversible, and the original spectrum is retained upon cooling. No such large shift is observed for TR upon heating, even above 70 °C.

Transient absorption spectra were recorded on a home-built multipass amplified Ti:sapphire apparatus, which produces 30 fs, 0.8 mJ pulses at the rate of 700 Hz and centered at 800 nm. Pump pulses were produced by TOPAS (Light conversion) centered at 530 or 560 nm, by mixing signal or idler with the fundamental. Another fraction of the fundamental pumped another TOPAS, which produced signal centered at 1450 nm. These NIR pulses were focused on a 3 mm BaF_2 flat to generate broadband white light probe. Probe intensity was directed to an imaging spectrograph (Oriel-Newport MS260i) equipped with a CCD (Andor technologies, Newton) through a three input fiber optic. Pump pulses (∼200 nJ, ∼150 μm radius, ∼2 GW/cm^2) were focused on the sample, flowing through a cell with quartz windows of 120 μm and 0.5 mm thickness. The optical density (OD) of the sample in the flow cell was ∼0.5. Sample temperature was controlled as described above.

RESULTS

The visible absorption peaks of all the proteins experimented on are presented in Figure 1, where TR is shown to have an absorbance maximum at 530 nm.[28] This is blue-shifted compared to the close structural homologue GR, whose highest absorptivity is at 545 nm,[43] and even more with respect to BR whose absorbance peaks at 565 nm.[44] The time corrected transient absorption data for TR irradiated at 530 nm and probed from 460 to 1030 nm is shown in Figure 2. As for previously studied MRPs,[45] the difference spectrum just after excitation consists of a ground state bleach contributing a negative band according to the spectrum in Figure 1, stimulated emission starting at ∼600 nm and extending to the red, and two excited state absorption bands, one centered at ∼500 nm and

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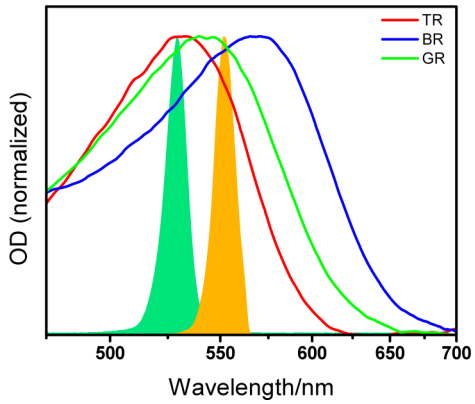

Figure 1. Absorption spectrum of bacteriorhodopsin, *Gloeobacter* rhodopsin and thermophilic rhodopsin. The pump pulse used to excite TR and GR is shaded in green, and the yellow shade shows the pump spectra exciting BR.

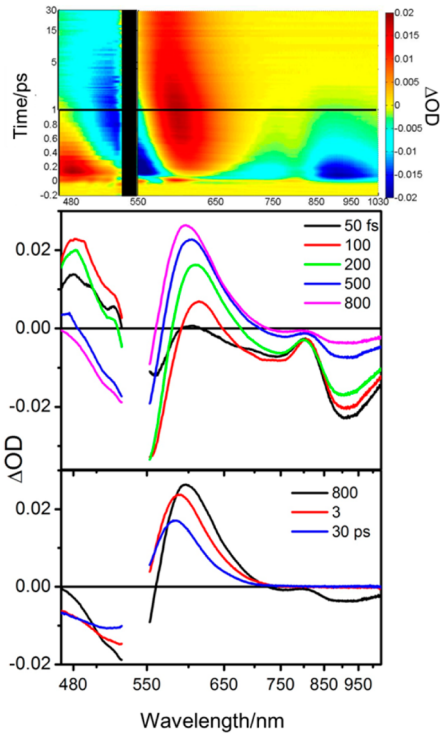

Figure 2. Transient absorption spectra of TR. The lower panels show the spectral cuts at different times. The black strip shades the region where the signal is masked by scatter from pump. Negative signal corresponds to ground state bleach and stimulated emission, while the positive signal corresponds to excited state or photoproduct absorption, as the case may be.

the other overlapping the emission giving rise to the dimple in the emission band at 770 nm.

As photoisomerization proceeds, all these bands decay, while the first ground-state photoproduct builds up and finally dominates the transient spectrum beyond the first picosecond of delay. The time dependent intensity profile (spectral cut) of this data at 955 nm, the peak of excited state stimulated emission, is compared with similar cuts obtained from samples of BR and GR in Figure 3A. As shown in previous studies, this spectral region is exclusively influenced by excited state activity and provides the most reliable measure of excited state decay.[8] It is evident that the TR excited state lifetime is significantly

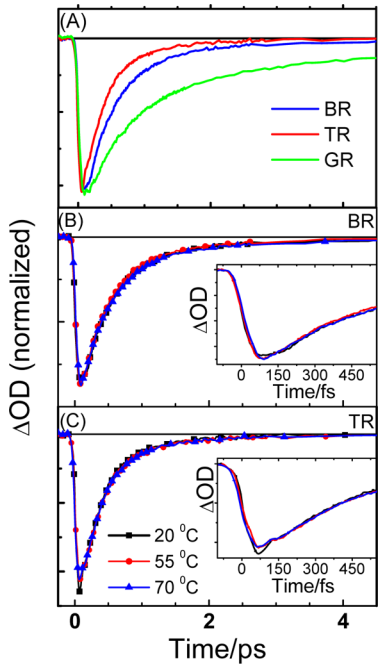

Figure 3. (A) Overlay of 20 °C stimulated emission traces for BR, TR, and GR. (B, C) Kinetic traces of BR and TR, respectively, probed at 955 nm at 20 (black), 55 (red), and 70 °C (blue). The insets in panels b and c are the same traces with the axis expanded up to 0.5 ps (500 fs).

shorter than that of BR and more than two times shorter than that of GR.

In order to quantify excited state decay kinetics, an average lifetime (τ) was calculated as follows (see derivation SIO):

$$\langle\tau\rangle = \int_0^\infty \left(\frac{dS_{\text{normalized}}}{dt}\right) t \, dt$$

where S is a normalized decay signal of the stimulated emission. This provides a weighted average of the excited state lifetime, which is model neutral and therefore less biased than other methods of defining IC kinetics. The results of this estimate are shown in Figure S2, giving values of 470 fs for TR, 740 fs for BR, and 1.5 ps for GR. These are the limiting values of lifetimes taking into consideration all routes of decay, with an estimated precision of within 10% of the nominal value.

As discussed below, the deviation from monoexponential excited state decay in MRPs has been extensively investigated both experimentally and theoretically.[19,45−48] In order to characterize such deviations in these experiments, the curves in Figure 3A were fit to a biexponential decay without y offset. The results are presented in Figure S3 and Table 1. The species associated spectra obtained by global analysis are consistent with these lifetimes reported here (Figure S4).

The amplitudes and decay times presented in the latter isomerization kinetics were recorded analogously at three

Table 1. Time Constants for Isomerization Rates in TR, BR, and GR Obtained from Biexponential Fit

	τ_1 (ps)	τ_2 (ps)	a_1	a_2
TR	0.36 ± 0.01	2.6 ± 0.8	0.96 ± 0.01	0.04 ± 0.01
BR	0.47 ± 0.01	2.8 ± 0.4	0.92 ± 0.01	0.08 ± 0.02
GR	0.77 ± 0.01	7.4 ± 0.2	0.77 ± 0.01	0.23 ± 0.01

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different temperatures, 22, 50, and 70 °C, for TR and BR (Figure 3B,C). BR is known to show structural changes in the protein at elevated temperatures.[38−40] Figure 3 shows the kinetic traces of BR and TR probed at 955 nm at the above-mentioned temperatures. Despite the recorded T dependence of sample absorption in BR (Figure S1), no temperature dependent changes in IC kinetics are observed in either of the two samples over this range. The excited state decay kinetics recovered by following stimulated emission at 955 nm are perfectly overlapping at the different temperatures, except at initial times post-excitation. These slight differences are assigned to low frequency modulations observed in these proteins due to coherent wave packet motions in skeletal modes of retinal.[49,50] Low frequency vibrations with a period of ∼170 fs, are observed for BR, in line with the earlier publications where these coherences were characterized extensively. These vibrations are more prominently observed at lower temperatures. The ∼90 fs period of corresponding vibrations in TR is thus almost two times shorter. GR, the structural analogue of TR, exhibits torsional modulations very similar to TR in frequency. These vibrations are present over the entire spectrum where excited electronic states are observed (Figure 4). Fourier power spectra of the residuals obtained

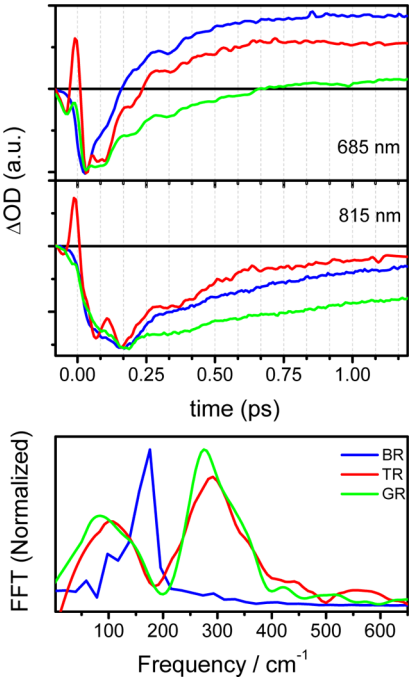

Figure 4. Low frequency vibrations in BR, TR, and GR. Probe wavelength of 685 and 815 nm are displayed in the upper two panels. FFT (Fast Fourier transform) power spectra of modulations isolated from the 815 nm data are presented in the bottom panel. FFT of modulations from BR are taken from ref 50.

from the data show a major peak centered at ∼300 cm^{-1} in TR and GR. Due to the extreme effort involved in extracting wave packet modulations, the power spectrum for BR was adopted from ref 50.

DISCUSSION

The salient observations for discussion are the rapid and exponential nature of isomerization in TR relative to other microbial proton pumps, the lack of any temperature dependence on IC kinetics in TR and in BR from room temperature to the verge of denaturation, and the torsional wave packet motions observed in TR, BR, and GR upon photoexcitation.

Isomerization Rates in TR. Isomerization rates are central to the photochemistry of both type I and II RPs.[51] Bovine rhodopsin and BR were both shown early on to photoisomerize with high efficiency and repopulate the ground state more than 10 times faster than retinal protonated Schiff base (RPSB) in solution, demonstrating what has been coined protein catalysis of RP photochemistry.[52−54] Over time, this aspect of RP photochemistry has taken on an importance of its own, particularly in the context of their serving as paradigms for rapid artificial photoswitches.[55] Study of numerous MRPs has however shown that these extreme rates are key neither to high quantum efficiency nor to efficient photon energy storage.[56,57] The subsequent time scales for biological utilization of this energy do not explain the necessity of such briefness either. Thus, efforts to clarify what determines the rapid rates of photoisomerization in some RPs remains a focus of attention in this field.

Defining the correct reference for the rate of TR isomerization is challenging. Since MRPs function by isomerizing from all-*trans* to 13-*cis*, all could in principle provide such a reference. However, in view of the structural alterations in the vicinity of the RPSB/counterion complex in pumps of other ions (Na^+, Cl^- etc.) or in sensory rhodopsins, we will restrict ourselves to comparison with other proton pumps.[9] Our BR sample was a suspension of fragmented purple membranes, while both GR and TR samples were expressed in *E. coli* as recombinant proteins and reconstituted in detergent (*n*-dodecyl-*b*-D-maltoside) solution. The consequences of this need comment before comparing the various data sets. Mounting evidence shows that IC rates change negligibly when tested in or outside the native membrane and that will hereafter be assumed for all studied samples.[58] This cannot be said about the response to elevated temperatures. Solubilized BR has been shown to degrade at lower temperatures than when incorporated in purple membrane.[31] Therefore, the correct reference for the intrinsic thermal stability would be that of the detergent stabilized state. In this respect the order of thermal stability is clearly TR > BR > GR, and TR is expected to be particularly temperature resistant due to its host's natural habitat.

In this limited context, the TR obviously isomerizes more rapidly than any other known proton pump. Before dealing with the significance of this, we must consider the quantum efficiency of isomerization, ϕ. As in the case of radiative vs observed lifetimes of a fluorophore, short lifetimes reflecting negligible reactive efficiency would be a trivial result. In the case of MRPs, it is the combination of efficiency and speed that is remarkable.[59,60] However, the quantum efficiency of isomerization in TR has not been determined. Nonetheless, we can employ the transient absorption data presented in Figures 2 and S4 to gain a rough estimate of the isomerization efficiency. The peak bleach signal of S_0 can provide a measure of the initially excited population. Accordingly the residual difference spectrum amplitude at ∼3 ps, representative of the first relaxed S_0 intermediate, can provide a lower limit of ϕ. Assuming an equal peak absorbance for "K" and "TR", which will underestimate ϕ somewhat, leads to an efficiency of at least 60% (Figure 5). Notice that for BR the ratio between the early ground state bleach and the ultimate "K" difference spectrum is only 0.44 while the known efficiency is close to 60%. This is

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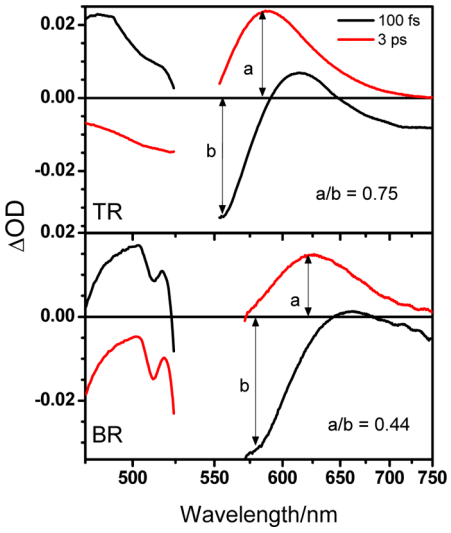

Figure 5. Relative ratio of reactant to product in TR and BR.

consistent with the known ratio of dipole strength between the two states and the shift in peak absorbance. The changes in peak absorption frequency and in transition dipole between the ground state and the photoproduct can vary from one protein to another. Given the coarseness of this estimate, all it proves is that ϕ is non-negligible and probably on par with or even higher than that of BR.

Thus, the rapid IC in TR is not a trivial effect of low yield but represents a significant reactive trait. After justifying the chosen references and estimating ϕ, assigning the cause of rapid isomerization in TR to thermal stability remains ambiguous. The three proteins tested here do indeed show a positive correlation between reaction rates and thermal stability. However, blue and green variants of proteorhodopsin (PR) have also been tested for thermal denaturation, and the latter, despite exhibiting much slower photoisomerization, is shown to be even more stable than solubilized BR.[30] This presents a prime reference system that goes against the trend above. This counter example is all the more significant since it involves a bacterial proton pump that is phylogenetically closer to TR than BR.[28] All that can be said in this context is that the one proton pump known to function in an organism adapted to extremely high temperatures, which exhibits heightened resistance to thermal denaturation even in detergent solution, undergoes unusually rapid IC, at least 30−40% faster than in bacteriorhodopsin (Figure 5). But no consistent trend connecting rapid IC with thermal stability in all tested proton pumps has emerged.

Thermal Effects on IC Dynamics. Various excited potential topologies have been suggested to explain isomerization dynamics of MRPs, particularly in contrast to Rh.[18,20,24,45,48,61] In the latter, continuous spectral evolution during isomerization signifies ballistic curve crossing to the ground state, culminating with coherently vibrating products.[13,12,62−64] In contrast MRPs exhibit a conserved excited state spectrum during isomerization, verified using three pulse pump−dump−probe methods.[65,47] Accordingly photoisomerization probably takes place gradually from a semi-stationary state, possibly due to phase-space barriers en route to reactive curve crossings. Deviation from monoexponential excited state decay is another prevalent characteristic of MRPs. It has been assigned in some cases to dispersive motions of excited state population possibly resulting in distinct reactive and unreactive channels.[66,67] Others have assigned this to inhomogeneous ground state structure.[45] The former has been challenged by the three pulse experiments cited above, which suggest that it is not a major mechanism in representative cases. As for the inhomogeneity, it is seldom tested since most methods for altering the degree of structural inhomogeneity will have other effects that are difficult to factor out. Temperature might be an exception. It is reasonable that altering protein temperature to the verge of melting should enhance the range of structural fluctuations in the irradiated sample.

It is for this reason that strict temperature invariance of IC dynamics both in TR and in BR is noteworthy (Figure 3). It challenges our understanding of both aspects of MRP photochemistry, that is, the delayed quasi-stationary excited state behavior and nonexponential isomerization. Clearly the delay in accessing the ground state does not depend strictly on pre-existing thermal energy in the retinal chromophore. This is demonstrated by cryogenic measurements of photoreaction in BR[68] and is in agreement with estimates for ground state barriers for isomerization relative to the ∼50 kcal/m of energy imparted by the absorbed photon.[57] Nonetheless, the possible requirement of extensive photon energy rearrangement for accessing reactive conical intersections poses a phase space barrier or bottleneck, which can produce delayed and apparently stochastic arrival at critical crossing geometries.[69]

Coarse estimates of the degree to which thermal internal motions should influence such dynamics can be derived by comparing the photon energy with the total internal vibrational energy of the retinal prosthetic group at room temperature and at 70 °C. The heat capacity of isolated retinal protonated Schiff base changes by ∼50 to 60 k_B going from 298 to 345 K, changing thermal energy content from 1.3 to 1.8 eV.[70] Inclusion in the protein must restrict some of the free motions contributing to this estimate. Accordingly a shift in total internal energy, including the 2.1 eV photon, from 3 to ∼3.5 eV might not be sufficient to influence dynamics, which probably are dominated by redirection of the same photon reorganization energy into reactive channels.

It is however hard to imagine how thermal agitation, which brings the BR to the brink of denaturation, has absolutely no effect on multiexponential decay, if it is indeed the hallmark of ground state structural inhomogeneity of the protein. Kinetic analyses of BR pump−probe signals have noted a clear improvement of fitting when extending beyond a single decay time scale, including results above for which biexponential fits are presented in the Supporting Information and in Table 1. If the nonexponential decay dynamics in BR were due to such fluctuations, nonexponential decay kinetics should become more pronounced at elevated temperature, particularly given the perturbation in steady state spectra. These results argue against structural inhomogeneity being the source of prevalent multiexponential decay kinetics in MRPs. Thus, both suggested interpretations of this attribute in MRP photochemistry have been seriously challenged, calling for future investigation of this point.

Torsional Coherences. Coherent wave packets launched by photoexcitation in low frequency torsional modes, first observed by Ye et.al.[49] in BR, are observed in our data for GR and TR as well. As in BR, the strongest signature of the resulting spectral modulations appears in the 700−800 nm range where excited state absorption and emission bands, and possibly weak contributions from ground state absorption, may

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overlap.[45] While this makes their analysis challenging in transient absorption, assignment to excited state vibrations has been verified by time-resolved fluorescence spectroscopy in RPSB as well.[71]

Two observations concerning these signatures are noteworthy. First is their diminishing amplitude with increased temperature, both in BR and in TR. In the case of BR, this might be assigned to temperature induced changes in absorption since the amplitude of modulations depends on the steepness of the shifting spectral bands. But in the case of TR where there is no sign of spectral alterations with increased temperature, this trend is not easily explained (Figure S5). If it were due to enhanced structural inhomogeneity as discussed above, it should also have impact on the steady state absorption, etc. Perhaps more interesting are the very significant differences in frequency of these modulation from one protein to another. Hou et al. showed that BR photoinduced torsional coherences are higher in frequency than those observed in RPSB, tentatively assigning this to a tighter restoring force on torsions due to a constraining protein environment.[49,50]

Investigation of other MRPs such as pharaonis halorhodopsin (pHR), or with superior signal-to-noise in BR shows this to be an oversimplified picture. First, equivalent coherences in pHR, for instance, are even lower in frequency than the free retinal chromophore.[65] Second, improved signal-to-noise proves these low frequency peaks to consist of a number of torsional motions, making the analysis in terms of a single frequency an oversimplification.[72,14] In the case of retinal PSB in solution,[50] harmonics of the fundamental frequencies were observed depending on the probe frequency chosen as expected for resonant detection of coherent wave packets.[73] Nonetheless, Figure 4 shows Fourier analysis of these modulations in three proteins, demonstrating a remarkable similarity in these signatures for TR and GR, both having prominent peaks at ~300 cm^{-1}. This value is significantly different from that observed for the protonated Schiff base of retinal in organic solvents, where these torsional frequencies are observed around 100 cm^{-1}.[50] Deviation from this value is observed for almost all known microbial rhodopsins. While these deviations are suggested to reflect variations in the protein specific microenvironment around the chromophore, their values, which are both positive and negative, have yet to be quantitatively explained. Along with the well-established values of ~80 cm^{-1} in pHR, 170 cm^{-1} in BR, and the near doubling in GR and TR, it appears that these signatures provide sensitive probes of the protein binding pocket, indicating the similarity of the sequence and accordingly the structure of the protein in both bacterial proton pumps TR and GR. This aspect of MRP pump−probe signals is under further investigation in our lab.

CONCLUSIONS

Photoisomerization rates of TR, a proton pump indigenous to the extreme thermophilic bacterium *Thermus thermophilus* JL-18, were recorded for the first time as a function of temperature in the range 20−70 °C. TR is found to have the fastest isomerization rate of any microbial proton pump, isomerizing 30% faster than BR. TR excited state also exhibits unusually monoexponential internal conversion kinetics. The isomerization process is estimated to proceed with high quantum efficiency of at least 0.6, indicating that this short lifetime is not a consequence of reduced isomerization yield. No temperature effects were observed on the primary photoinduced events of TR or BR from room temperature to the verge of thermal denaturation. This observation rules out nonexponential isomerization in BR to be ascribed to structural inhomogeneity in the irradiated sample. Coherent torsional wave-packets launched by impulsive photoexcitation of TR have a marked similarity to its structural analogue *Gloeobacter* rhodopsin. However, these are significantly different from those observed for other proton pumps like BR or pHR. These observations show that these modulations, now recorded in several microbial rhodopsins as well as in the retinal chromophore in solution, serve as sensitive probes of the protein binding pocket and its interaction with the prosthetic group.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b05002.

Temperature dependent absorption changes of BR, temporal analysis, and the rationale behind estimation of average life times of BR, GR, and TR (PDF)

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Notes

The authors declare no competing financial interest.

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