Single-Shot Sub-microsecond Mid-infrared Spectroscopy on Protein Reactions with Quantum Cascade Laser Frequency Combs

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Supporting Information

ABSTRACT: The kinetic analysis of irreversible protein reactions requires an analytical technique that provides access to time-dependent infrared spectra in a single shot. Here, we present a spectrometer based on dual-frequency-comb spectroscopy using mid-infrared frequency combs generated by quantum cascade lasers. Attenuation of the intensity of the combs by molecular vibrational resonances results in absorption spectra covering 55 cm⁻¹ in the fingerprint region. The setup has a native resolution of 0.3 cm⁻¹, noise levels in the μOD range, and achieves sub-microsecond time resolution. We demonstrate the simultaneous recording of both spectra and transients of the photoactivated proton pump bacteriorhodopsin. More importantly, a single shot, i.e., a single visible light excitation, is sufficient to extract spectral and kinetic characteristics of several intermediates in the bacteriorhodopsin photocycle. This development paves the way for the non-invasive analysis of enzymatic conversions with high time resolution, broad spectral coverage, and minimal sample consumption.

Time-resolved studies of reactions in the condensed phase are of outstanding relevance to chemistry and biology. Important processes whose subsecond kinetics are key for future developments include the fixation of CO₂ into fuels,¹ water oxidation in photosynthesis,² or the folding of proteins.³ Understanding of the underlying mechanisms presents scientists with a chance to develop revolutionary means of energy conversion and treatments for diseases such as Alzheimer’s or Parkinson’s.⁴ Infrared and Raman spectroscopy allow for studying minute yet relevant structural changes in an analyte because of their sensitivity to single proton movements or hydrogen bond rearrangements. Time scales from femtoseconds to microseconds are addressed by pump–probe methods,⁵⁻⁷ whereas the important time range from nanoseconds to tens of milliseconds is covered by infrared techniques including step-scan FTIR,⁸ tunable laser,⁹ dispersive infrared,¹⁰ and resonance Raman spectroscopy.¹¹ Unfortunately, these techniques require a considerable number of acquisitions to collect a full set of time-dependent spectra.

Therefore, natural photoinduced processes that are either thermally reversible within an amenable time (e.g., microbial rhodopsin)¹² or returnable to their initial state by photons or oxygen¹³,¹⁴ have been studied in detail (Figure 1). However, several highly important natural processes are irreversible, such as the responses of visual rhodopsin¹² and the B₁₂-dependent CarH receptor,¹⁵ or are non-repeatable such as water splitting by photosynthetic reaction centers.¹⁶,¹⁷ Others are only slowly repeatable, including the recovery of phototropin, which regulates plant growth toward the light.¹⁸ In particular, caged compounds are a convenient but irreversible approach for the fast initiation of enzymatic reactions for time-resolved studies.¹⁹ All these processes have in common that their

Received: June 5, 2018
Accepted: August 6, 2018
Published: August 6, 2018
comprehensive analysis requires excessive, often prohibitive, amounts of sample for repetitive experiments.

Frequency-comb spectroscopy has enabled the direct observation of time-resolved broadband spectra, since its characteristic minimum acquisition time is linked to intrinsic properties of the frequency combs (see below). Fiber-based frequency combs were employed to achieve sub-millisecond, high-resolution gas spectroscopy. Extending these sources into the mid-infrared spectral range remains challenging, and the search for more efficient comb generation mechanisms such as microresonators is ongoing. The discovery of an electrically pumped frequency-comb source that is based on a semiconductor quantum cascade laser drastically simplified mid-infrared dual-comb spectroscopy. The chip-scale spectrometer that, with its parallel measurement of hundreds of wavelengths with sub-microsecond time resolution, was applied to a single-shot kinetic analysis of a biological photo-reaction.

# EXPERIMENTAL SECTION

Sample Preparation. Wild-type bacteriorhodopsin was obtained by homologous expression from *Halobacterium salinarum* (strain S9). *Halobacterium salinarum* was grown in 4.3 M NaCl, 80 mM MgSO₄, 27 mM KCl, 10 mM sodium citrate, and 10 g L⁻¹ peptone medium, pH 6.5, for 8 days and lysed by addition of distilled water. Fragments of purple membrane (PM) containing bacteriorhodopsin were isolated by fractionated centrifugation according to Oesterhelt and Stoeckenius. Either 3 or 12 µL of PM suspension with 530 µM bacteriorhodopsin was slowly dried on a BaF₂ window and rehydrated to the original volume with 1 M KCl and 50 mM K₂HPO₄/KH₂PO₄ buffer, pH 7.2, 9.0, or 6.0, respectively. A second BaF₂ window was applied to prevent evaporation of the droplet during 30 min of rehydration. Afterward, the sample was sealed using vacuum grease, and the path length was reduced to 4 or 16 µm to achieve an absorbance of 0.8 or 2.2, respectively, at 1650 cm⁻¹. The temperature of the samples was kept constant at 17 °C by a circulating water bath. After an equilibration time of at least 30 min, the sample was light-adapted by exposure to the 532 nm actinic laser.

Frequency-Comb Spectroscopy. The experiments were conducted using a tabletop dual-frequency-comb spectrometer. The emission of two frequency combs is combined using a table-top dual-frequency-comb spectrometer. The 0.3 cm⁻¹ spacing of the frequency-comb modes is ideal for solid- or fluid-phase measurements and also allows for sub-microsecond time resolution. The 0.3 cm⁻¹ resolution per channel (Keysight U5303A). A germanium detector (Ultra100, Quantel) with a pulse duration of 10 ns at a repetition rate of 2 Hz. The laser beam was expanded to a diameter of 1.5 cm using a biconcave lens with a focal length of 250 mm resulting in an intensity of 12 mJ/cm² at the sample. For a time range of 5 ms prior to the laser pulse, dark spectra were recorded, averaged, and used as a reference for the calculation of light-induced difference spectra. Subsequently, the response of the sample was monitored for 15 ms.

Step-Scan FTIR Spectroscopy. Step-scan measurements were performed on an IFS 66v spectrometer (Bruker) equipped with a DC-coupled photoconductive mercury cadmium telluride (MCT) detector at a spectral resolution of 4.5 cm⁻¹. An infrared band-pass filter (Laser Components) was used to block stray light and restrict the spectral range to 1974–988 cm⁻¹. For excitation, 532 nm pulses with a duration of 10 ns were generated by an optical parametric oscillator (Opta) pumped by the third harmonic of an Nd:YAG laser (Quanta-Ray 130–10, Spectra Physics) with an intensity of 5.2 mJ/cm² at the sample. The 10 Hz repetition rate of the laser was reduced to 2 Hz by an optical shutter (LSTXY, nm Laser Products). In total, 449 mirror positions were recorded with 4 coadditions and 1000 equidistant time slices of 5 µs up to 5 ms. Reference spectra were recorded for 173 µs, before excitation, and used to calculate light-induced difference spectra. Samples had a path length of 4 µm and were exchanged after a maximum of 10 776 excitations. For the final spectra, 11 experiments were averaged with 44 coadditions in total corresponding to ~20 000 excitations.

Data Analysis. The digitized beating signal was analyzed using MATLAB (Mathworks). The 20 ms time traces were cut into time slices of 4× the length of the required time resolution, with subsequent time slices overlapping by 75% of their length. This procedure is justified by a “loss” of 75% of the signal information by applying a flat-top window to each time slice. In spite of the overlaps, the evaluation led to fully uncorrelated signals of adjacent time slices. After Fourier transformation, the equidistance of the beating signals was used to automatically detect the peak positions of individual beating signals. The amplitude at the peak position of the heterodyne spectrum retrieved from the signal detector was then normalized to the amplitude of the same time slice retrieved from the reference detector. The ratio of signal to reference detector amplitudes was then taken as the instantaneous value of the transmitted laser intensity. The pretrigger intensity (here, the first 5 ms of each acquisition) was averaged and used as a background measurement. The post-trigger intensities normalized by this background represented the time-resolved difference spectra.

Further, the time-resolved spectra were analyzed using MATLAB. For the dual-comb spectra, 6, 14, and 25 adjacent lines were averaged and weighted according to their line intensity, resulting in a resolution of 2.0, 4.5, and 8.0 cm⁻¹, respectively. For the transients, 14 or 31 lines were averaged and weighted according to their line intensity. To increase the signal-to-noise ratio, dual-comb data taken after 130 µs and step-scan data recorded after 125 µs were averaged on a logarithmic time scale.

A weighted global fit was applied using a kinetic model of sequential first-order reactions with four intermediates. The weighting factors were calculated from the variance of the reference spectra along the wavenumber dimension. Nonlinear fits were performed with the program SolvOpt for MATLAB (The Mathworks). From the concentration profiles, the species-associated difference spectra (SADS) were calculated.
RESULTS AND DISCUSSION

Establishing a Dual-Comb Spectrometer. The dual-comb system (Figure 2A) contains two free-running quantum cascade laser (QCL) frequency combs centered at a wavelength of $\sim 1220$ cm$^{-1}$ and spanning 70 cm$^{-1}$ (Figure 2B, inset). They overlap in the spectral range from 1185 to 1240 cm$^{-1}$ resulting in dual-comb absorption spectra covering 55 cm$^{-1}$. The continuous wave output power is about 700 mW, causing an average power per comb tooth of >2 mW. The two frequency-comb sources are combined using a 50:50 beam splitter and attenuated with neutral density filters. One combined beam is focused on a high-bandwidth HgCdTe reference detector. The other combined beam is transmitted through the sample, passed through a germanium visible beam block, and detected by a second HgCdTe detector. A heterodyne beat spectrum (Figure 2B) results from the mixing of the two frequency-comb lasers, as schematically shown in the top panel of Figure 2C. The short cavity length of QCLs causes an average power per comb tooth of >2 mW. The two frequency-comb sources are combined using a 50:50 beam splitter and attenuated with neutral density filters. One combined beam is focused on a high-bandwidth HgCdTe reference detector. The other combined beam is transmitted through the sample, passed through a germanium visible beam block, and detected by a second HgCdTe detector. A heterodyne beat spectrum (Figure 2B) results from the mixing of the two frequency-comb lasers, as schematically shown in the top panel of Figure 2C. The short cavity length of QCLs causes an average power per comb tooth of >2 mW. The two frequency-comb sources are combined using a 50:50 beam splitter and attenuated with neutral density filters. One combined beam is focused on a high-bandwidth HgCdTe reference detector. The other combined beam is transmitted through the sample, passed through a germanium visible beam block, and detected by a second HgCdTe detector. A heterodyne beat spectrum (Figure 2B) results from the mixing of the two frequency-comb lasers, as schematically shown in the top panel of Figure 2C. The short cavity length of QCLs causes an average power per comb tooth of >2 mW.
cm$^{-1}$, as commonly used for time-resolved FTIR spectroscopy. In this case, we observe a peak-to-peak variation on the order of 100 $\mu$OD throughout the high-intensity range of the spectrum, while the noise level is about 10 times higher in the low intensity range from 1205 to 1215 cm$^{-1}$.

**Analysis of a Biological Photoreaction.** The present research focuses on the well-understood kinetics of the transmembrane protein bacteriorhodopsin from *Halobacterium salinarum*, which binds a retinal as chromophore in a seven-$\alpha$-helical fold (Figure 2A). Bacteriorhodopsin acts as a light-driven proton pump and undergoes a photocycle starting with the isomerization of the retinal (Figure 3A,B). It has been previously extensively characterized regarding its intermediates and the proton-pumping mechanism using time-resolved FTIR spectroscopy, among other methods. Bacteriorhodopsin is an exceptionally stable protein, which withstands more than 100,000 excitations, thus allowing for extensive averaging. Here, we demonstrate that even a single excitation can elucidate its various photocycle states.

We investigated the photocycle of bacteriorhodopsin using a well-hydrated film embedded in its native environment as purple membrane patches at pH 7.2, 1 M KCl and 17 °C, close to physiological conditions. After recording 5 ms of reference transmission, the photocycle of bacteriorhodopsin was initiated using a 10 ns laser pulse at 532 nm for excitation with an intensity of 12 mJ/cm$^2$. The spectral evolution was monitored for another 15 ms, and absorption difference spectra were calculated. The experiment was repeated at 2 Hz, and 500 averages were performed. In the spectral range accessible by the dual-comb spectrometer, we detected two marker bands of retinal at 1189 (+) cm$^{-1}$ and 1200 (−) cm$^{-1}$ (Figure 3C), which have been assigned previously to changes in the C–C...
stretches and coupled CCH rocks of the retinal chromophore. Four intermediates ($t_1 = 12 \mu s$, $t_2 = 140 \mu s$, $t_3 = 3.3 ms$, $t_4 > 12 ms$) were resolved between 2.2 $\mu s$ and 15 ms by performing a spectrally weighted global fit with sequential reactions (Figure S1, Supporting Information). Characteristic changes over time resulted from averaging raw data according to the maximal concentrations of the intermediates from the global fit (Figure 3D). Among others, the transient loss at 1189 cm$^{-1}$ reflects the protonation of the retinal linkage in the M intermediate.

Reference measurements were acquired with a step-scan FTIR spectrometer under the same conditions as those of dual-comb spectroscopy, except for a lower excitation intensity of 5.2 mJ/cm$^2$ (Figures 3E and S2, Supporting Information). The resulting species-associated difference spectra (SADS) were assigned to intermediates $K_L$, $L$, $M$, and $N$ in this spectral range (Figure 3E). A direct comparison of the M spectrum between dual-comb and step-scan spectroscopy shows excellent agreement, if the same (Blackman–Harris) windowing is applied (Figure 3F, blue and black trace). The spectrum exemplifies that the quality of the dual-comb recording is as high as that of FTIR spectroscopy after the same data treatment. The dual-comb data was also evaluated using a higher spectral resolution of 2 cm$^{-1}$, resolving a negative band at 1226 cm$^{-1}$ in the spectrum of the M intermediate (Figure 3F). This high resolution would require a severe increase in repetitions when using step-scan FTIR spectroscopy (here from 19,756 to 41,492 shots), whereas it is inherent in dual-comb spectroscopy. Typically, such high resolution is not required to resolve the majority of signals in biological samples, but it may provide valuable information. Accordingly, the band at 1226 cm$^{-1}$ has been observed in high-resolution experiments at 220 K but we assert that it has not been resolved previously in time-resolved experiments.

**Single-Shot Spectra and Kinetics.** Strikingly, a single shot of the experiment clearly resolved all characteristic profiles of the intermediates without a baseline correction or any other manipulation of the data (Figure 3G). To illustrate that the selection of the single shot was representative, the standard deviation of difference absorbance over 50 single shots was included (Figures 3G and S3, Supporting Information). It is important to note that this time-resolved experiment is the first single-shot analysis of a protein reaction in the mid-IR spectral range. Previous experiments have succeeded in resolving single-shot kinetics at selected wavenumbers. Here, we covered a broad spectral range comprising multiple absorption bands. The coverage is only limited by the current status of QCL comb technology.

Next, the limits of the time resolution were evaluated. At the expense of lower spectral resolution and higher noise, the time resolution limit $T_{\text{res}} = 2\Delta t_{\text{rep}}$ was overcome, and a resolution of 320 ns was achieved. A linear time trace (Figure 3H, inset) shows the decay of the $K_L$ intermediate. From the raw data, a spectrum at 320 ns was isolated without performing any global analysis, which represents the pure $K_L$ intermediate (Figure 3H). The spectrum clearly differs from the mixture of $K_L$ and $L$ obtained at 2.2 $\mu s$. Evidently, even the submicrosecond intermediates of the photocycle can be elucidated with the dual-comb approach with only few repetitions of the experiment.

Finally, reaction kinetics at the maxima of the difference bands were evaluated from 2.2 $\mu s$ to 15 ms covering almost 4 orders of magnitude in time (Figure 4). A good agreement was obtained with results from step-scan spectroscopy (Figure 4A). The time range of the dual-comb spectrometer can even be extended $>15$ ms by applying larger memory electronics. An advantage of the considerably higher intensity of the QCL emission as compared to the globar in the FTIR spectrometer is espoused by its capacity to strongly increase the limited path length of below 10 $\mu m$ in H$_2$O, which facilitates the application of flow systems without degrading the sample (Figure S4, Supporting Information). Moreover, we show that identical kinetics but significantly higher signal can be obtained from a sample having 4-fold longer path lengths (Figures S5 and S6, Supporting Information). To demonstrate the sensitivity, the effect of pH on the photocycle was examined (Figure 4B). The increase in pH from 6.0 to 9.0 led to a decrease in rate constants after 100 $\mu s$, which was quantitatively analyzed and assigned, applying global fits to the average of 500 acquisitions (Figure S7, Supporting Information). Formation and decay of the N intermediate is characterized by an internal reprotonation of the retinal Schiff base linkage and an uptake of a cytoplasmic proton by Asp96, respectively. Therefore, the
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centrization in agreement with literature (at higher pH, both M and N are accumulated).35,43

Most importantly, the kinetics was observed even in a single shot (Figure 4C). Application of a global fit to the single shot at pH 6.0 enabled us to successfully identify three out of four time constants (τ2 = 84 μs, τ1 = 1.3 ms, τ0 = 8.5 ms) (Figure S8, Supporting Information). The volume employed for these single-shot kinetic experiments was 12 μL at a sample concentration of 530 μM. The volume actually probed by the IR light, however, amounts to only 200 nL as derived from the beam diameter of 4 mm and a path length of 16 μm. Thanks to the single-shot performance, very low sample consumption might therefore be achieved in the case of irreversible reactions.

CONCLUSIONS AND OUTLOOK

We have presented a QCL-based dual-comb spectrometer that can be used to analyze the kinetics of reactions in a single shot. The experiment delivers full spectra every 320 ns for a duration of 20 ms. While the native spectral resolution of the dual-comb system is well below the line width of condensed-phase spectra, a lower resolution can be used to improve the signal-to-noise ratio. At native resolution, an RMS measurement noise of 1 mOD at 100 μs time resolution in a single shot and 50 μOD in 500 shots is observed, which reduces to 400 μOD in a single shot and as low as 15 μOD in 500 shots at 4.5 cm⁻¹ resolution. By this, we were able to reproduce all of the characterics of the intermediates Kp, L, M, and N of the bacteriorhodopsin photocycle in the spectral range of 1185–1240 cm⁻¹. The single-shot measurement capability makes vibrational spectroscopy of irreversible reactions with sample volumes as low as 200 nL possible.

Dual-comb spectroscopy’s application is not limited to photoreceptive systems such as bacteriorhodopsin but can be extended to other reactions triggered by external perturbations such as jumps in electric potential or temperature. Moreover, channel or enzymatic activity can be artificially initiated for time-resolved studies by the modern tools of optogenetics.47 Above all, the high temporal resolution in combination with the broad-band mid-infrared measurement performance opens a plethora of applications where short measurement times are required, such as in-line quality control in a production environment or high-throughput screening in drug development. Given its potential to integrate the light source into an all-solid-state solution, the present work represents an important cornerstone for a broadband laser-based lab-on-a-chip device,48 enabling smart monitoring solutions in environmental or personalized medicine.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b02531.

Global fit of results from dual-comb spectroscopy and step-scan FTIR spectroscopy, representation of a single shot, influence of the probe light exposure on the sample, absorption spectra of samples with different path lengths, dual-comb experiments on samples with different path lengths, global fit of results at different pH values, global fit of a single-shot experiment (PDF)

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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