Spatiotemporal Resolution of Conformational Changes in Biomolecules by Combining Pulsed Electron–Electron Double Resonance Spectroscopy with Microsecond Freeze-Hyperquenching

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ABSTRACT: The function of proteins is linked to their conformations that can be resolved with several high-resolution methods. However, only a few methods can provide the temporal order of intermediates and conformational changes, with each having its limitations. Here, we combine pulsed electron–electron double resonance spectroscopy with a microsecond freeze-hyperquenching setup to achieve spatiotemporal resolution in the angstrom range and lower microsecond time scale. We show that the conformational change of the Cβ-helix in the cyclic nucleotide-binding domain of the Mesorhizobium loti potassium channel occurs within about 150 μs and can be resolved with angstrom precision. Thus, this approach holds great promise for obtaining 4D landscapes of conformational changes in biomolecules.

INTRODUCTION

The function of biomolecules is intimately linked to their structure and dynamics. Often, effector-triggered conformational changes are key to protein function. Membrane-spanning proteins such as G protein-coupled receptors or ion channels, which are particularly challenging for structural biology,1,2 exist in active and inactive conformations. The transition between active and inactive form is triggered by ligand binding or physical cues such as changes in membrane voltage,3–6 absorption of light,7,8 or mechanical forces.9 These conformational changes happen on different length and time scales ranging from angstrom to nanometers, and picoseconds to seconds.10 X-ray crystallography, NMR spectroscopy, and electron microscopy greatly advanced our knowledge about structures and their dynamics. Here, we introduce a combination of pulsed electron–electron double resonance spectroscopy (PELDOR or DEER)12–14 with so-called microsecond freeze-hyperquenching (MHQ) as a complementary technique (MHQ/PELDOR) to achieve a 4D conformational landscape from the initial to the final conformational state with high spatiotemporal resolution.

PELDOR yields ensemble distributions of distances between electron-spin centers in frozen samples in the range of 1.5 to 16 nm with angstrom precision.15 In biomolecules, spin centers can be introduced via site-directed spin labeling (SDSL) of, for example, cysteine residues in proteins by means of nitroxide spin labels.16 Combining SDSL and PELDOR, the structures and conformational changes of large proteins, oligonucleotides, and protein/oligonucleotide complexes have been studied in solution, in membranes, or even whole cells. Although PELDOR provides information on the conformational ensemble present at the freezing point, it is blind to the time scales and sequence of conformational events. However, coupling SDSL/PELDOR with fast freeze-quench techniques may permit taking snapshots along the trajectory of a conformational change and provide access to the time domain with temporal resolution only limited by the mixing and freezing kinetics. The time scale of protein dynamics ranges from femtoseconds for bond vibrations via nano- and microseconds for movements of α-helices and β-sheets, up to seconds or even hours for folding and assembly of multi-subunit proteins. Ligand-induced conformational changes are of particular interest, as they trigger important cellular reactions, and the rate-limiting steps are often unknown. To follow such movements in proteins, mixing and freeze-quenching should be completed within microseconds. While...
typical freeze-quench setups operate on a millisecond time scale, an MHQ device can reach minimal aging times of roughly 100 μs. MHQ combined with continuous wave (CW) electron paramagnetic resonance (EPR) has been used to examine the binding kinetics of the azide/methmyoglobin system, the lifetime of catalytic intermediates, the refolding of cytochrome oxidases, and the electron-transfer rates in the respiratory complex I. Two previous studies have employed freeze-quench/PELDOR to test the distance distribution width with respect to the freezing time. The transport time \( t_\text{f} \) is set by the jet velocity and the distance between the mixer orifice and the cold-plate. Its lower limit is given by a safe minimal distance between the orifice and the cold-plate of about >2 mm (SI Section 1.1). Microsecond quenching requires high linear flow rates (up to 200 m s\(^{-1}\)) achieved by HPLC pumps. Small uniform jet diameters are obtained by operating the mixer and the cold-plate under a vacuum hood. The vacuum prevents jet breakup and ensures that experimental and theoretically expected transport times match. The actual transport time \( t_\text{f} \) as demonstrated by laser Doppler anemometry, is only <10% shorter than the value calculated from flow rates. The shortest aging time \( t_\text{a} \) achieved was 82 μs (Table S1).

**RESULTS**

Calibration of the MHQ Device. The reaction kinetics between equine heart metmyoglobin (MetMb) and azide (Na\(_3\)) was employed to calibrate the MHQ aging times. In the apo state, the Fe(III) ion in the heme group is in the high-spin state (\( S = 5/2 \), abbreviated \( hs \)), and binding of azide switches it to the low-spin state (\( S = 1/2 \), abbreviated \( ls \)). \( hs \)- and \( ls \) Fe(III) give rise to an apparent axial \( g_{ax} = g_{yy} = 5.8 \), \( g_{zz} = 2.0 \) and orthorhombic EPR spectrum \((g_{xx} = 2.8, g_{yy} = 2.2, g_{zz} = 1.8)\), respectively, which allows following the progression of the reaction by CW EPR spectroscopy (Figure 2).

The reaction gradually progressed with the aging time (Figure 2c,d, Extended Data Figure 1, SI Section 9.1). The signal intensities of \( hs \) Fe(III) at \( g_{xx} = g_{yy} = 5.8 \) decreased with \( t_\text{a} \) whereas the intensities of the \( ls \) state \((g_{xx} = 2.8 \text{ and } g_{yy} = 2.2)\) concomitantly increased (Figure 2c). The fraction of \( hs \) and \( ls \) state trapped in the frozen sample at an aging time \( t_\text{a} \) can be deduced from the peak-to-peak intensities of the respective signals in the CW EPR spectra (Figure 2c and SI Section 4.5).

These fractions were fitted by two exponentials \( y = ae^{-kt_1} + be^{-kt_2} \) with pseudo-first-order rate constants \( k_1^\prime = 19 \text{ 669} \text{ s}^{-1} \) and \( k_2^\prime = 1358 \pm 189 \text{ s}^{-1} \) (Figure 2d). These rate constants \( k \) translate into second-order rate constants \( k_1 = 26 225 \text{ ± } 7208 \text{ M}^{-1} \text{ s}^{-1} \) and \( k_2 = 1811 \pm 253 \text{ M}^{-1} \text{ s}^{-1} \), which are in good agreement with previous accounts (Table S2).

The biphasic behavior may be attributed to different reactions occurring in parallel, e.g., binding of N\(_3\) and HN\(_{3}\) or temperature drifts depending on the length of the jet. The dispersion of the \( y \)-values (coefficient of variation = SD/mean) (Figure 2d) was maximally 17% for \( t_\text{a} = 82 \text{ μs} \) and ranged between 0.6% and 8.0% for all other data points. Thus, for our purpose, reliable aging times as short as 82 μs and up to 668 μs can be obtained with the MHQ device, yet the operational range is much larger (<20 ms).

**EXPERIMENTAL SECTION**

The MHQ device is based on a prototype reported by de Vries’ group (Figure S1). It consists of a rotating aluminum cold-plate, a vacuum hood, and two syringes connected by tubing to a four-jet tangential micromixer. The protein and ligand solutions are injected from syringes via tubing into the micromixer. The mixture is ejected from the micromixer as a free-flowing thin jet (diameter: 20 μm) that is freeze-quenched within microseconds on a rotating cold-plate. The micromixer is mounted on a robotic swivel arm that allows vertical and horizontal movements. Vertical movement assures that the entire cold-plate surface is optimally used for freezing; horizontal movement sets the distance between micromixer and cold-plate and, thereby, the aging time, \( t_\text{a} \).

The aging time \( t_\text{a} \) consists of three components: the mixing time \( t_\text{m} \) (residence time in the micromixer), the transport time \( t_\text{f} \) (time-of-flight in the jet), and the quenching or freezing time \( t_\text{q} \) (SI Section 1.1).

The time \( t_\text{a} \) is determined by the mixer volume and the flow rate during mixing. The time \( t_\text{f} \) depends on the jet diameter as well as the heat conductivity of the aqueous sample and the cold-plate material. The diameter of the jet, known from the orifice diameter, and the volumetric flow rate yield a \( t_\text{m} \) of 1 μs and a \( t_\text{f} \) of approximately 40 μs. The transport time \( t_\text{f} \) is set by the jet velocity and the distance between the mixer orifice and the cold-plate. Its lower limit is given by a safe minimal distance between the orifice and the cold-plate of about >2 mm (SI Section 1.1). Microsecond quenching requires high linear flow rates (up to 200 m s\(^{-1}\)) achieved by HPLC pumps. Small uniform jet diameters are obtained by operating the mixer and the cold-plate under a vacuum hood. The vacuum prevents jet breakup and ensures that experimental and theoretically expected transport times match. The actual transport time \( t_\text{f} \) as demonstrated by laser Doppler anemometry, is only <10% shorter than the value calculated from flow rates. The shortest aging time \( t_\text{a} \) achieved was 82 μs (Table S1).
187 E336R1 with a narrow distribution widths. In addition, the pair R254R1/
188 distance changes. The amino-acid pair E289R1/I340R1 separated (1)
189 is on the C
187-helix; (2) the distance distributions of apo and
186 states range between 2 and 8 nm to facilitate high-quality
185 PELDOR data; and (3) the distance distributions should be
184 narrow (<1 nm full width at half-height, fwhh) and well
183 separated (Δr > 0.6 nm) to facilitate the identification of
distance changes. The amino-acid pair E289R1/I340R1
182 (where R1 refers to the nitroside-labeled cysteine) was
181 particularly promising due to its large Δr of ~1.8 nm and its
180 narrow distribution widths. In addition, the pair R254R1/
179 E336R1 with a Δr of only ~0.3 nm was selected to gauge the
178 limitations of the method.

**Figure 2.** Reaction between MetMb and NaN₃ converting Fe(III)
from the ls to the ls state. (a) Lewis structures of the ls and ls state.
For clarity, only the porphyrin ring of MetMb is shown. Orbital
175 diagrams indicate the occupation of t₂g and e₉g orbitals in the
octahedral ligand field for either the ls or ls state. (b) Continuous-
174 wave X-band (9.4 GHz) EPR spectra recorded at 20 K of MetMb in
the ls state (left) and the ls state (right). In the spectrum of the
ls state, a residual of the ls Fe(III) state due to incomplete conversion
is marked by (#); a resonator background signal is marked by an asterisk
(*). (c) Stack plot of CW X-band EPR spectra recorded from samples
undergoing different aging times rₙ. (d) Fraction of the apo and holol
state as a function of rₙ. The fractions have been derived from the
signal amplitudes of ls Fe(III) and ls Fe(III) in the CW EPR spectra
(SI Section 4.5) and are given as mean (full circle) ± SD (error bars)
of triplicates. Solid lines: fit using two exponentials (y = ae⁻ktᵢ +
bexp) to experimental data. To ascertain pseudo-first-order kinetics,
NaN₃ was used in 1000-fold excess. Postmixing concentrations:
[MetMb] = 0.75 mM, [NaN₃] = 0.75 M. Note that at rₙ = 0, about 3%
l ls Fe(III) MetMb is present.

**Controls.** We subjected these constructs to several controls
to assess whether the spin label and the EPR sample
preparation significantly affect the protein structure, the ligand
binding, or the conformational change. First, we studied
whether two native cysteine residues (C263, C331) of the
CNBD were accessible for cysteine-reactive reagents and
would interfere with distance determinations between
exogenous spin labels. In the apo state, incubation of wild-
type (wt) CNBD with Ellman’s reagent modified approx-
imately one cysteine per CNBD monomer, and the resulting
protein was no longer able to bind cAMP. Therefore, we
decided to remove the endogenous cysteines by site-directed
mutagenesis. Of the different constructs, C263S/C331L, which
was suggested by the software CUPSAT (Cologne University
Protein Stability Analysis Tool), displayed the lowest Kᵣ
d value for 8-(2-[-7-nitro-4-benzofurazanyl]aminoethylthio)-
adenosine-3',5'-cyclic monophosphate (8-NBD-cAMP) (103 ±
27 mM). All mutants studied here are based on this
cysteine-free C263S/C331L mutant.

Second, as spin labeling in high yields and quantitative
cAMP removal required unfolding and refolding of the protein,
we assessed the potential impact of unfolding/refolding on the
distance distribution by also purifying and labeling the CNBD
without unfolding (SI Section 2.8). The most probable
194 distances and distribution widths of the apo and holol state of
193 construct E289R1/I340R1 prepared via unfolding/refolding or
in the native state agree well (Figure S8). Moreover, CW EPR
spectra recorded at room temperature suggest that the local
flexibility of the spin labels is similar for the two protein
197 samples (Figure S9). This result indicates that the protein
structure is not altered by unfolding and refolding.

Third, we probed how the R1 side chain affects binding of cAMP
and 8-NBD-cAMP to the CNBD by independent
199 techniques: the dissociation constants Kᵣ were determined
200 either in a stopped-flow setup using the NBD fluorescence or
201 by isothermal calorimetry (SI Section 3). Although the
202 Kᵣ constants were altered, values were <10 μM, similar to the
203 binding constants of cAMP and cGMP for the CNG channels
204 in olfactory neurons and photoreceptors, respectively (Table
205 S5).

Fourth, we tested whether the structures of the apo and holol
206 states are preserved upon labeling and freezing in the MHQ, by
207 comparing the experimental (PELDOR) distance distributions
208 of the apo and holol states with those predicted by mtslWizard
209 or MMM on the basis of the NMR structures. We have
210 chosen the NMR structures instead of the crystal structures
211 because crystal packing effects may have altered the structure
212 and some of the amino-acid residues are not resolved in the
213 crystal structure. The experimental distance distributions of the
214 mutated and labeled CNBD are highly similar compared to the
215 distance distributions generated in silico by means of
216 mtslWizard or MMM from the NMR structures. This comparison shows that the global
217 protein structure has not been disturbed significantly.

Fifth, we compared the distance distributions of samples which
were rapidly quenched in the MHQ (τᵣ = 82 μs) with samples
slowly immersed in liquid nitrogen (freezing time ~1.5 s). Rapid
freezing by MHQ does not allow for sufficient time to
relax to the thermodynamic energy minimum at the freezing
point, and, compared to slow freezing, a broader conformational
total ensemble may be trapped. This can lead to differences in the
shape and width of the distance distributions depending on the
freezing conditions. Here, we find that distributions

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using difference-distance maps generated with the in-silico spin-
labeling program mtslWizard (SI Section 5 and Figure S6).

The selection was based on three criteria: (1) one labeling site
is on the Cₙ-helix; (2) the distance distributions of apo and
holo states range between 2 and 8 nm to facilitate high-quality
PELDOR data; and (3) the distance distributions should be
narrow (<1 nm full width at half-height, fwhh) and well
separated (Δr > 0.6 nm) to facilitate the identification of
distance changes. The amino-acid pair E289R1/I340R1
(where R1 refers to the nitroside-labeled cysteine) was
particularly promising due to its large Δr of ~1.8 nm and its
narrow distribution widths. In addition, the pair R254R1/
E336R1 with a Δr of only ~0.3 nm was selected to gauge the
limitations of the method.
broaden in fast-frozen samples, apart from the apo state of E289R1/I340R1, where the intensity ratios of the peaks at 4 nm are altered (Figure S10).

Finally, we examined whether the addition of 20% deuterated ethylene glycol (EG-d_{6}) as cryoprotectant affects the EPR properties of the spin system. We observed that the phase-memory time T_{2} of the spin label increases from 2.5 to 3.2 $\mu$s upon adding EG-d_{6}, which enables longer time windows for the dipolar evolution in the PELDOR experiment and improves the signal-to-noise ratio (Figure S11 and Table S6). In addition, the background correction of PELDOR time traces is improved, and thus more reliable distance distributions are obtained. Beyond this, the distance distributions with and without EG-d_{6} are very similar, from which we conclude that the protein structure is not affected by the cryoprotectant.

In conclusion, these controls show that the label and rapid MHQ freezing does not alter the global structure or the function of the protein.

**Assessing the Conformational Change.** The apo state sample of the MloK1 CNBD was mixed in the MHQ, with buffer only. The holo state, which is the cAMP/CNBD complex, was first formed outside of the MHQ by adding cAMP and then mixed with buffer in the MHQ. The final protein and cAMP concentrations were 150 M and 15 mM, respectively. Of note, the mixer had been only. The final protein and cAMP concentrations were 150 M and 15 mM, respectively. Of note, the mixer had been designed to not contain cAMP.

The apo and holo state of E289R1/I340R1 display a bimodal distance distribution with both modes falling within the envelope predicted by mtslWizard (Figure 3). We reason that the bimodality is caused by interactions of the label rotamers with the protein, which are difficult to predict by in-silico methods (Figure S7). The change in the most probable distances, $\Delta r = −1.9$ nm, between apo and holo state agrees with the mtslWizard prediction. For the R254R1/E336R1 construct, experimental and predicted distance distributions for the apo and holo state also match within the error margin of mtslWizard (0.4 nm). Based on the PELDOR background validation (Figure 3), the peak for the apo state at 2.5 and 2.8 nm for constructs E289R1/I340R1 and R254R1/E336R1, respectively, may indicate a small contribution of the holo state, whereas the minor peaks at longer distances are artifacts. Overall, the distance change of $\Delta r = −0.4$ nm between the apo and holo state of R254R1/E336R1 is in good agreement with the mtslWizard prediction. Due to the particularly small distance change and large overlap between the distance distributions of apo and holo, this construct indeed illustrates the resolution of our method.

Next, we examined the kinetics of the conformational change during the transition from the apo to the holo state by varying $t_a$ from 82 to 498 $\mu$s. After 1:1 mixing the protein sample (200 $\mu$L) in the MHQ with cAMP-containing buffer (500 $\mu$L), final protein and cAMP concentrations were 150 $\mu$M and 15 mM, respectively. Of note, the mixer had been first conditioned with an excess of cAMP-containing buffer (300 $\mu$L) before the apo protein entered the mixer. This precaution ascertains that the protein sample was not partially mixed with buffer that did not contain cAMP.

As can be gleaned from Figure 4a,d, the time traces and the distance distributions change with $t_a$. The time-resolved changes in the distance distributions for E289R1/I340R1 show that with increasing $t_a$, the apo and holo state populations respectively decreased and increased stoichiometrically (Figure 4b,c and Extended Data Figure 2, SI Section 9.2). However, neither a gradual shift of the most probable distance from the apo to the holo state nor additional peaks along the reaction coordinate were observed, suggesting that structural intermediates are not detected.

The fraction of apo and holo state was quantified by deconvolution of the PELDOR time traces (SI Section 4.7). A plot of the fractions of apo and holo state versus $t_a$, was fitted by a monoexponential function:

$$y = y_0 + A e^{-kr}$$

yielding a rate constant $k$ of 7398 $\pm$ 1179 s$^{-1}$ for E289R1/I340R1 and of 7508 $\pm$ 867 s$^{-1}$ for R254R1/E336R1. All regression parameters are collected in Table S3. Interestingly, the k values are highly similar, indicating that the underlying...
process monitored by MHQ/PELDOR is independent of the labeling site on the Cα-helix. In addition, construct R254R1/E336R1 illustrates that even changes as small as 0.4 nm of strongly overlapping distance distributions can be resolved. Because our experiments monitor ensemble averages, the Cα-helix movement of individual molecules, which supposedly is rapid and stochastic, is not resolved in the MHQ/PELDOR experiment. Indeed, molecular dynamics (MD) simulation suggests that the helix movement takes only a few nanoseconds, which is much faster than the experimental rate constant k (Figure S13 and Extended Data Figure 5, SI Section 9.3). Therefore, we scrutinized whether the kinetics reflects rate-limiting cAMP binding. To this end, MHQ samples were prepared at a \( t_a \) of 303 \( \mu \)s. The protein concentration after 1:1 mixing in the MHQ was in each case 150 \( \mu \)M, whereas the final cAMP concentration varied between 0.5 and 15 mM, equivalent to a CNBD/cAMP ratio ranging from 1:3 to 1:100. The background-corrected PELDOR time traces, the corresponding distance distributions, and the calculated fraction of apo and holo state versus cAMP concentration are shown in Figure 5 (Extended Data Figure 3, SI Section 9.2).

At cAMP concentrations larger than 10 mM (\([cAMP]\) / \([CNBD]\) ~6)), the fractions are independent of the cAMP concentration, confirming saturation of the CNBD with cAMP. This demonstrates that diffusion-controlled ligand binding under the MHQ/PELDOR conditions is not rate-limiting and that the rate-limiting step occurs further down the apo-to-holo pathway. This conclusion was confirmed by measuring the
The dwell time of the apo-ligand complex is different for each individual protein molecule, resulting macroscopically in a distribution of dwell times. Such a distribution of dwell times is also compatible with MD simulations, which show that dwell times of the apo-ligand complex derived from MD trajectories are exponentially distributed (Figure S14 and SI Section 9.3). For short aging times \( t \), in relation to the individual dwell time, only a small number of protein molecules can undergo the conformational transition. For longer aging times, the probability increases that protein molecules populate the holo state. Thus, the time constants determined for E289R1/I340R1 and R254R1/E336R1 reflect the average dwell times for the Cα-helix movement or the binding kinetics themselves. This picture agrees with recent atomistic simulations, which revealed "prebinding" of the ligand to different surface sites, followed by induced-fit conformational motions of the binding pocket and entropic barriers to ligand binding as the rate-limiting steps. We note that this concept neither rules out conformational motions during the first ligand binding steps, that, however, are below the PELDOR resolution, nor claims that, for the apo-ligand complex, the ligand is already positioned at its final binding site. It does imply, though, that the second step is independent of concentration.

MHQ/PELDOR is able to extract dwell times from conformational rearrangements, further illustrating the power of this technique to study ligand-triggered protein kinetics. Comparing the PELDOR-derived dwell times with those inferred from MD reveals an 18-fold difference (Figure S14). This discrepancy may be attributed to two factors. First, the temperature of the solution jet is not precisely known, but likely below room temperature, and may decrease gradually over the jet length. By contrast, the MD simulation temperature was 300 K. Thus, the experimentally determined dwell times are expected to be longer. Second, because the entire conformational change is a stochastic multistage process whose duration exceeds the simulation time of 3.5 µs, only the first step(s) en route to the holo state is monitored in the MD simulation. Thus, the MD simulation probably underestimates the overall dwell time. This combination of overestimation of dwell times in the experiment and underestimation in the MD simulation may explain the discrepancy.

For this proof-of-concept study, we used high concentrations and larger volumes to obtain high-quality data at short measurement times (60 nmol protein, 200 µL, i.e., 300 µM per point at a signal-to-noise ratio (SNR) between 70 and 100 K/\( \nu \) and a time trace length of 2.8 µs). To demonstrate the sensitivity of MHQ/PELDOR, we also used protein amounts as small as 7.5 nmol (75 µM, 100 µL) per time point, which still yielded a good SNR of 25 for a measurement time of 8 h (SI Section 8). Thus, the protein amount, measurement time, and SNR can be similar to that of typical Q-band PELDOR measurements. Sample consumption can be reduced even further when working at higher EPR frequencies such as W-band.

Spatiotemporal resolution has also been achieved with other methods, each having its own strengths and limitations and often being complementary to each other. For example, a fluorescently labeled ligand, such as 8-NBD-cAMP, versus a spin label addresses two different observables: fluorescence spectroscopy probes the environment of the cAMP ligand and thus reports on the crossing of barrier 1 in Figure 6 (aqueous solution vs complexation in the hydrophobic protein), whereas...
CONCLUSION

In conclusion, the MHQ/PELDOR approach holds great potential for following conformational changes in large biomolecules with spatiotemporal resolution on the angstrom and microsecond time scale. In our proof-of-concept study, we have determined the mean dwell time for a helix movement triggered by a small ligand. MHQ/PELDOR promises to temporally resolve both dwell times and slower conformational transitions that happen in the >100 μs range. On a final note, MHQ/PELDOR is by no means restricted to proteins: It might be useful to follow conformational changes in any biomolecule, provided that these changes can be triggered by an external event such as ligand binding and that they proceed on a time scale of >82 μs. As the MHQ device is operated under a vacuum hood, quick changes of pressure or temperature are presumably difficult to implement. However, we envision to use the MHQ as a fast-freezing device and trigger folding events or conformational changes by light using photolysis of caged compounds, photoswitches, or phototriggers.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c01081.

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