Published in final edited form as:

J Phys Chem B. 2018 May 24; 122(20): 5218–5224. doi:10.1021/acs.jpcb.8b03354.

Probing Cytochrome *c* Folding Transitions upon Phototriggered Environmental Perturbations Using Time-Resolved X-ray Scattering

Dolev Rimmerman^{†,‡}, Denis Leshchev^{†,‡}, Darren J. Hsu[†], Jiyun Hong[†], Baxter Abraham[‡], Robert Henning[§], Irina Kosheleva[§], and Lin X. Chen^{*,†,||}

[†]Department of Chemistry, Northwestern University, Evanston, Illinois 60208, United States

[‡]Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716, United States

[§]Center for Advanced Radiation Sources, The University of Chicago, Chicago, Illinois 60637, United States

^{||}Chemical Sciences and Engineering Division, Argonne National Laboratory, Argonne, Illinois 60439, United States

Abstract

Direct tracking of protein structural dynamics during folding–unfolding processes is important for understanding the roles of hierarchic structural factors in the formation of functional proteins.

Using cytochrome *c* (cyt *c*) as a platform, we investigated its structural dynamics during folding processes triggered by local environmental changes (i.e., pH or heme iron center oxidation/spin/ligation states) with time-resolved X-ray solution scattering measurements. Starting from partially unfolded cyt *c*, a sudden pH drop initiated by light excitation of a photoacid caused a structural contraction in microseconds, followed by active site restructuring and unfolding in milliseconds. In contrast, the reduction of iron in the heme via photoinduced electron transfer did not affect conformational stability at short timescales (<1 ms), despite active site coordination geometry changes. These results demonstrate how different environmental perturbations can change the nature of interaction between the active site and protein conformation, even within the same metalloprotein, which will subsequently affect the folding structural dynamics.

Graphical Abstract

*Corresponding Author l-chen@northwestern.edu, lchen@anl.gov.

[‡]D.R. and D.L. contributed equally.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.8b03354.

Detailed experimental methods, SAXS characterization results, solvent subtraction, global analysis routines, pH-jump magnitude estimation, and uncertainties in TRXSS data (PDF)

Notes

The authors declare no competing financial interest.

INTRODUCTION

Protein structure–function relationships are known to be sensitive to environmental conditions, such as temperature, pH, and redox media.^{1–4} In vivo, the interplay of environment, structure, and function provides a regulatory mechanism for controlling biological functions via conformational gating or ligand binding.^{5–8} Therefore, understanding protein structural dynamics in response to a particular environmental perturbation is a key in elucidating the protein functional mechanisms.

Protein folding dynamics typically span multiple timescales and can range from nanoseconds to milliseconds for fast folding proteins.⁹ Traditional time-resolved structural biology methods, such as stopped flow or continuous flow mixers, typically involve a dead time of over 100 μ s and consume large sample volumes, making them unsuitable for tracking dynamics on short timescales.^{2,10,11} In contrast, pulsed laser-based methods can initiate reactions on timescales as short as femtoseconds, making them suitable for observing short-lived intermediates.^{12,13} Although significant progress has been made during past decades, the laser-based approach faces an inherent limitation because naturally occurring light-driven proteins are rare^{14,15} and an intrinsic chromophore is needed to drive the dynamics and probe the structural changes. Here, we present a “pump-probe” protein folding–unfolding study of cytochrome *c* (cyt *c*) which circumvents the requirement for an intrinsic chromophore by utilizing laser excitation to perturb the protein environment by pH-jump or photoreduction and probe the resulting structural changes with time-resolved X-ray solution scattering (TRXSS). TRXSS is capable of direct tracking of protein tertiary structural dynamics, which gives an important advantage over spectroscopic methods that only indirectly infer global protein kinetics.¹⁶ Therefore, it provides an alternative method to optical transient absorption (OTA) or UV-CD in tracking folding kinetics of tertiary structures.

Cyt *c* is a heme metalloprotein that takes part in the mitochondrial electron transport chain, as well as in the apoptosis process;¹⁷ its dual functions are closely tied to the interactions between the peptide backbone and heme iron center.^{18–21} In the native folded state of cyt *c*, the heme iron has a pseudo-octahedral coordination with axial ligation to the N atom in H18 and the S atom in M80, respectively.^{18,19} The Fe–S (M80) ligation, however, is known to be weak and readily dissociates in the absence of protein structural constraints, resulting in heme iron axial ligations with other residues or small molecules.^{18,20,22} In the presence of

3–4 M guanidine HCl (GuHCl), the protein unfolds and the native M80 ligation of the oxidized heme iron (Fe(III)) is replaced by a non-native ligand, depending on the pH of the environment. At a neutral pH, the native M80 ligand is replaced by one of the deprotonated histidines, either H26 or H33 ($pK_a \approx 5.5$); while at $4 < \text{pH} < 5.5$, M80 is replaced mainly by H_2O ,^{20,23–26} whereas H18 still remains intact ($pK_a \approx 2.8$ for H18 in oxidized cyt *c*).

In addition, the heme iron coordination geometry also highly depends on the iron oxidation state.^{20,27} Under the same denaturing conditions and at neutral or low pH, the reduced heme iron (Fe(II)) favors the native M80 ligation to adopt the folded protein conformation.¹⁸ Although the differences in conformational stability have been previously attributed to a stronger Fe(II)–S bond in M80, some recent studies have indicated that this bond is in fact weaker compared to the corresponding oxidized Fe(III) form.^{22,23} These revelations have emphasized the role of protein conformation in stabilizing the local active site ligation.

The differences in protein conformational stabilities under various heme iron ligation states were exploited by several OTA studies to reveal the cyt *c* active site dynamics using pH-jumps and photoinduced reduction of heme iron via electron transfer (ET).^{18–20,27} The results of the previous OTA studies^{20,27} are summarized in Figure 1. When the oxidized heme iron Fe(III) was reduced to the Fe(II) state by the ET process, the resulting refolding dynamics were found to be particularly sensitive to the heme ligation state. When H_2O replaces M80 as an axial ligand at $\text{pH} < 5.5$, biphasic kinetics were observed (reactions 2–3 in Figure 1), but when M80 axial ligation is replaced by deprotonated H26 or H33 at a neutral pH, monophasic kinetics were reported (reaction 4 in Figure 1). Therefore, the rate-determining step of the folding process at $\text{pH} \approx 5.5$ is the deligation of H26/H33 (reaction 4 in Figure 1), whereas at $\text{pH} < 5.5$, it is the ligation of the M80 residue to the five-coordinated heme iron (reaction 3 in Figure 1). The protonation-induced H26/H33 deligation dynamics (reaction 1 in Figure 1) were shown to follow biexponential kinetics on millisecond timescale, originating from the parallel deligation processes from two possible H26/H33 ligations with slightly different lifetimes. However, despite the progress in understanding the active site dynamics, OTA is only able to provide limited and indirect information about time-dependent changes in secondary and tertiary structures of the protein. Specifically, questions remain regarding the protein conformation at each ligation state, the degree of unfolding, and the role of structural constraints in active site dynamics. In this study, we trigger protein conformation changes in cyt *c* by environmentally inducing heme ligation state change as shown in Figure 1. Protein refolding dynamics were investigated under two different triggering conditions with separate measurements—(1) histidine protonation via a photoinduced pH-jump (reaction 1) and (2) heme iron reduction via photoinduced ET (reactions 2–4). Both experiments utilized TRXSS to directly observe the resulting structural evolution of the protein.

EXPERIMENTAL SECTION

Sample Preparation—pH-Jump TRXSS and Static Small-Angle X-ray Scattering Experiments.

The samples were prepared by a procedure adapted from previously published methods.²⁷ Horse heart cyt *c* was purchased from Sigma-Aldrich and used without any further

purification. For pH-jump experiments, the protein was dissolved to a concentration of 2 mg/mL in a solution of 3.2 M GuHCl and 8 mM *o*-nitrobenzaldehyde (O-NBA) to make ~20 mL volume samples. Nitrogen was bubbled through a pure buffer bubbler and flowed over the sample reservoir during the experiments. pH was adjusted to the desired value by adding drops of 1 M NaOH and HCl solutions and verified by a pH meter. All samples were filtered using a 0.2 μ m filter prior to use. For all experiments, the samples were used immediately after preparation. Buffer heating TRXSS experiments were carried out on buffer solutions containing all components at the same concentration except the protein (GuHCl, O-NBA).

Sample Preparation—ET TRXSS.

The samples were prepared by a procedure adapted from previously published methods.²⁰ Horse heart cyt *c* was dissolved to a concentration of 6 mg/mL in solutions containing 3.2 M GuHCl, 1.25 mM Ru(bpy)₃, and 50 mM sodium phosphate buffer to make ~20 mL volume samples. Nitrogen was bubbled through a pure buffer bubbler and into the sample reservoir during the experiments. A drop of polypropylene glycol (PPG) was added to the samples to reduce foaming while bubbling nitrogen. All samples were filtered using a 0.2 μ m filter prior to TRXSS experiments. pH was adjusted to either pH = 7 or pH = 4 by using drops of 1 M NaOH and HCl solutions and verified by a pH meter. Buffer heating TRXSS experiments were carried out on buffer solutions containing all components at the same concentration except the protein (sodium phosphate, GuHCl, Ru(bpy)₃, PPG).

TRXSS—pH-Jump Experiments.

pH-jump TRXSS experiments were carried out at BioCARS 14-ID-B beamline at the advanced photon source (APS). Details of the X-ray scattering setup and generic TRXSS pump-probe data acquisition methodology at BioCARS have been previously published.^{4,16,28} The samples were delivered by a syringe pump into a custom built, temperature-controlled flow cell.⁴ The sample was excited by 7 ns laser pulses with 300 nm wavelength, which is suitable for exciting proton release from O-NBA.²⁹ The measurements were performed at 25 °C. The full experimental details are available in the supporting information

TRXSS—ET Experiments.

ET TRXSS experiments were carried out at BioCARS 14-ID-B beamline at the APS. The sample volume was delivered into a standard capillary flow cell by a syringe pump. The [Ru(bpy)₃]²⁺-containing sample was excited by picosecond laser pulses with 480 nm wavelength in accordance with previous optical studies.²⁰ The measurements were performed at room temperature. The full experimental details are available in the supporting information.

RESULTS AND DISCUSSION

Photoinduced pH-jump TRXSS experiments were carried out to explore the structural dynamics upon protonation of unfolded cyt *c* (reaction 1). The pH-jump was achieved by excitation of O-NBA, a photoacid generator that releases a proton in its excited state and is hence commonly used in triggering pH-sensitive dynamics.^{27,30–32} O-NBA is known to form *o*-nitrosobenzoic acid (pH $\approx$ 3.5) with quantum efficiency of ~0.5 following

photoexcitation with UV light.³² The photoinduced proton release is completed by ~20 ns following excitation, making it suitable for inducing dynamics in protein systems for time-resolved studies.⁹ Under the excitation conditions of the experiment (300 nm, 600 μJ per pulse, power density of ~32 mJ/mm² at the focal spot, and O-NBA concentration of 8 mM, see supporting information), it is expected that the concentration of released protons should be ~3–4 mM, a value which is limited by the solubility of the photoacid rather than laser power.³³ We experimentally estimated $\text{pH} \approx 1$, from an initial value of 5.5 to ~4.5 following the excitation (see supporting information). The evolution of the structural signal was tracked by TRXSS on timescales of 1 μs to 80 ms utilizing previously established pump-probe methodology.^{16,28}

The pH-jump-induced TRXSS differential signals contain contributions from both the protein structural dynamics and solvent hydrodynamic responses due to heating by the absorption of laser pulses. These contributions were disentangled by fitting TRXSS signals from protein-free solution, which were obtained in a separate measurement, to the TRXSS differentials from samples containing cyt *c* with O-NBA at $q > 0.5 \text{ \AA}^{-1}$. An example fit of the solvent heating signal to the differential TRXSS of cyt *c* and O-NBA is shown in Figure 2a. The comparison clearly shows that the signals at $q < 0.5 \text{ \AA}^{-1}$ are not associated with solvent heating but protein structural dynamics. On the basis of the laser intensity and the sample absorptivity used in the experiment, we conclude that the laser heating induces a solvent temperature change corresponding to ~1 K (see supporting information), a value observed in typical optical pH-jump experiments.³³

The protein-associated TRXSS signals, obtained by subtraction of the solvent contribution, are shown in Figure 2c. The observed protein-associated difference signals evolve from a weak positive signal, observed at early time delays (<300 μs), to more pronounced signals, composed of a positive feature at $q < 0.05 \text{ \AA}^{-1}$ and a negative feature at higher angles (e.g., $0.05 < q < 0.20 \text{ \AA}^{-1}$) at later time delays (>3 ms). A standard global analysis approach, based on singular value decomposition, was used to extract the number of transient species involved in the evolution of the signal (see supporting information for details).^{34–40} The analysis shows the existence of only two significant components, the signals associated with short time delays (the intermediate protonated species, I_{H}^{+}) and the signals at long time delays (the unfolded protonated species, U_{H}^{+}). The time-dependent changes in the species populations, as well as the solution temperature dynamics obtained from the solvent subtraction procedure, are shown in Figure 2d. The species-associated difference scattering patterns obtained from the global analysis procedure are shown in Figure 2e.

From the global analysis, it was found that the U_{H}^{+} state rises in about 4.0 ± 0.5 ms after the pH-jump and reaches a plateau by ~10 ms. The signal remains unchanged at even longer time delays, indicating that any structural changes due to the 1 K temperature rise are negligible, as any heating-induced scattering signal would diminish as the temperature recovers (Figure 2d). We attribute the U_{H}^{+} species sustainable up to 100 ms to the final protein conformation adopted in response to the deligation of protonated H26/H33 groups from heme and replacement by H_2O ligation. To further support this assignment, the time-resolved signal at 20 ms time delay, which represents the maximum TRXSS signal for U_{H}^{+} , was compared with structural differences derived from the static scattering data taken for cyt

c in solutions with pH = 4 and pH = 5.1 (see Figure 2b). The good agreement between the static difference and the time-resolved data confirms that the observed TRXSS signal in the small-angle X-ray scattering (SAXS) region is due to the structural rearrangements in cyt *c* induced by the change in pH. Qualitatively, the increase in the forward scattering observed at $q < 0.05 \text{ \AA}^{-1}$ arises from electron density increase due to the H₂O ligation, as well as an increase in solvent accessibility, whereas the negative feature in the signal around $0.1 \text{ \AA}^{-1}$ indicates the expansion of the protein.^{4,36,40} The latter is further supported by the Guinier analysis of the static data recorded at pH values of 4 and 7, which provides corresponding radii of gyration of ~ 35 and $\sim 30 \text{ \AA}$, respectively (see supporting information for details). Therefore, the H26/H33 deligation causes further unfolding of cyt *c*, which has not been directly observed previously in the OTA experiments.²⁷

The signal associated with an intermediate species, I_{H^+} , observed at 5–300 μs shows a positive differential signal mainly in the wide-angle X-ray scattering region. However, TRXSS signals recorded at earlier time delays ($< 1 \mu\text{s}$) did not show any protein-associated signal, despite a low signal-to-noise ratio, which suggests that the I_{H^+} state population rises on the timescale of several microseconds and then levels off (see supporting information). A signal matching these timescales has not been previously observed in OTA experiments, suggesting that it is unlikely to be attributed to heme active site dynamics. Furthermore, the overall absence of signal in the forward scattering region indicates that the scattering contrast between the protein and the buffer remains unchanged in this state. The positive feature indicates a slight contraction of the protein, which likely arises due to the disappearance of longrange Coulombic interactions between charged side groups exposed to the solvent that become protonated upon the pH-jump.³¹ The timescale of this process, a few microseconds, therefore reflects the time required for the protein backbone chain to adapt the new conformation via diffusion.

The pH-induced millisecond structural dynamics of cyt *c* agree with the active site dynamics revealed by OTA results from Small and co-workers, with the exception of showing single exponential kinetics rather than biexponential kinetics.²⁷ Specifically, the TRXSS-derived partial unfolding kinetics signal fits well to a lifetime of $4.0 \pm 0.5 \text{ ms}$, whereas OTA reportedly showed lifetimes of 3.7 ms (H26) and 13.1 ms (H33).²⁷ The two processes observed from OTA dynamics were attributed to the mixture of two His residues bound to the heme, which have different kinetic barriers for deligation. In the case of TRXSS-derived dynamics, we suggest that the changes in the overall protein structure are dominated by only one of the two species (H26). Regardless, the similarity between static and TRXSS-derived signals implies that TRXSS captures all global structural changes in the protein and that these are complete by 20 ms.

In addition to the investigation of pH-jump-induced structural dynamics, we also investigated the early structural dynamics of ET-induced cyt *c* folding (reactions 2–4 in Figure 1) by TRXSS (see Detailed Experimental Methods in the supporting information). ET is initiated by photoexcitation of $[\text{Ru}(\text{bpy})_3]^{2+}$ acting as a sensitizer and electron donor to give one electron to reduce the heme iron from Fe(III) to Fe(II). As detailed above, the protein with the oxidized heme Fe(III) becomes unfolded under the experimental denaturing conditions where the Fe–S(M80) ligation is lost; however, in the protein with the reduced

heme Fe(II), the protein adopts the folded state in which the Fe–M80 bond is restored. TRXSS data was taken following photoreduction from two initial pH conditions, pH = 7 and pH = 4. OTA studies have previously shown that ET is complete within 1 μ s of excitation, and back-ET (i.e., return to the ground state) is complete on several millisecond timescale, which limits the time window available for tracking the dynamics.²⁰ For this reason, the ET TRXSS experiments are focused on time delays between 1 μ s and 1 ms. OTA results suggest that H26/H33 ligands should mostly remain ligated to the heme on these short timescales (neutral pH), whereas H₂O would deligate to form a five-coordinated heme structure (low pH).²⁰

The comparison of the heme iron reduction-induced TRXSS difference signals associated with the proteins at the two starting pHs is shown in Figure 3a. Recorded TRXSS data were processed similarly to the pH-jump data presented above, with the solvent subtraction performed by using the TRXSS data recorded from photoexciting protein-free [Ru(bpy)₃]²⁺ solution as the signals for heating (see supporting information for details). The qualitative assessment of scattering difference curves, as well as a quantitative comparison including the experimental uncertainties (see supporting information for details), clearly demonstrates that at pH = 4, difference signals arising due to structural changes are already present by 500 μ s, whereas no major structural signals were observed for pH = 7 solution. The stability of the protein conformation at a neutral pH on short timescales following the heme iron reduction agrees well with the OTA results,²⁰ which observed that the active site dynamics occur at significantly longer timescales than those accessible by utilizing [Ru(bpy)₃]²⁺ photoreduction.

For the TRXSS data taken at pH = 4, the negative signal observed at the forward scattering region ($q < 0.05 \text{ \AA}^{-1}$) is attributed to the loss of electron density corresponding to the loss of H₂O ligation and formation of the reduced five-coordinated state of heme. The signal may also contain contribution from rearrangement in the solvation shell of the protein due to charge redistribution caused by H₂O deligation. Notably, the absence of the signal at higher q values suggests that the overall protein conformation remains stable during the transition from oxidized (Fe(III)) six-coordinated state to reduced (Fe(II)) five-coordinated state. To assess the timescale of H₂O deligation from cyt *c* at low pH, we have inspected the time series of the protein-associated TRXSS signals (Figure 3b) and investigated the time dependence of the total integrated signal at $q < 0.05 \text{ \AA}^{-1}$. The analysis shows that the structural change appears on sub-ms timescales in agreement with the previous OTA studies by Winkler and co-workers.²⁰ These observations suggest that the use of Ru(bpy)₃ photoreducing agents coupled with TRXSS is suitable for investigation of redox protein dynamics on microsecond timescales. Other studies utilizing alternative reducing mechanisms, such as NADH or pulsed radiolysis, have observed additional microsecond dynamics at neutral pH, which are associated with a specific protein subpopulation that can undergo reduction by a solvated electron or guanidine radical.^{41,42} These additional dynamics were not observed in the current work or the OTA work by Winkler and co-workers.^{20,43} For this reason, we suggest that either Ru(bpy)₃ does not efficiently reduce the specific subpopulation that can undergo fast folding and that this effect is uniquely observed in experiments where reduction is induced by solvated electrons (or radicals) or that the signal from this population is smaller than the detection limit in the current experiment.

Future experiments with alternative reducing agents, such as NADH, may provide further information regarding these different populations.

Beyond kinetic analysis, the TRXSS technique also carries an additional advantage to other kinetics experimental techniques, such as OTA and UV-CD, as it can also be used for advanced structural analysis of intermediates.^{16,40} Such analysis can be carried out by low-resolution shape reconstruction or rigid body modeling, though, this advanced analysis is not required for extracting kinetics. However, these modeling methods are not readily applicable to unfolded proteins, even in static scattering experiments, as the unfolded proteins are subject to conformational ensemble heterogeneity.⁴⁴ As an alternative to rigid body modeling, molecular dynamics simulations could be used for simulating the ensemble of unfolded structures. However, in the case of metalloproteins, this approach is precluded as modern force fields lack the capability of correct description of interactions between metal ion and possible ligands and remains a problem for experimental analysis.⁴⁵ Establishing the correct force field parameters, as well as their validation, is out of scope of the current work and will be published separately in the future.

CONCLUSIONS

In conclusion, our study shows that the combination of TRXSS structural probe with indirect excitation through photoinduced pH-jumps and photoreducing agents provides additional insights to optical characterization. In particular, TRXSS experiments are sensitive to both the active site dynamics and the global structural rearrangements in the protein, whereas the latter is not readily available from OTA experiments. This advantage of TRXSS allowed us to directly observe the formation of a previously unseen intermediate state in pH-jump experiments, which does not affect the active site dynamics directly and therefore could not be seen with OTA. More generally, our study, utilizing photoinduced pH-jumps and photoreduction, joins our previous study of temperature-induced structural dynamics in expanding the capabilities for direct observation of protein structural dynamics in nonphotoactive systems by TRXSS.⁴ With these new methodologies of TRXSS coupled to pH-jumps, T-jumps, and ET, it will be possible to investigate protein function in real time, as these reaction initiation methods are mechanistically similar to those employed in vivo. Overall, all three reaction initiation methods greatly expand the applicability of TRXSS for investigation of dynamics in nonphotoactive proteins, making it possible to perform detailed studies of biologically relevant processes with high structural sensitivity and high temporal resolution at the same time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Health, under contract no. R01-GM115761. B.A. acknowledges support from the U.S. Department of Energy (DOE), Office of Science Graduate Student Research program, administered by the Oak Ridge Institute for Science and Education, managed by ORAU under contract number DE-SC0014664, as well as from the U.S. DOE Office of Science, Office of Basic Energy Science, under award number DE-SC0016288. This research used resources of the APS, a U.S. DOE Office of Science User

Facility operated for the DOE Office of Science by Argonne National Laboratory under contract no. DE-AC02-06CH11357. Use of BioCARS was also supported by the National Institute of General Medical Sciences of the National Institutes of Health under grant number R24GM111072. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Time-resolved setup at Sector 14 was funded in part through collaboration with Philip Anfinrud (NIH/NIDDK). We would also like to acknowledge Guy Macha (BioCARS) for his assistance in designing the sample holder. Portions of this work were performed at the DuPont-Northwestern-Dow Collaborative Access Team (DND-CAT) located at Sector 5 of the APS. DND-CAT is supported by Northwestern University, E.I. DuPont de Nemours & Co., and The Dow Chemical Company. Data was collected using an instrument funded by the National Science Foundation under award number 0960140.

REFERENCES

- (1). Zhang X-X; Jones KC; Fitzpatrick A; Peng CS; Feng C-J; Baiz CR; Tokmakoff A Studying Protein-Protein Binding through T-Jump Induced Dissociation: Transient 2D IR Spectroscopy of Insulin Dimer. *J. Phys. Chem. B* 2016, 120, 5134–5145. [PubMed: 27203447]
- (2). Sato D; Ohtomo H; Yamada Y; Hikima T; Kurobe A; Fujiwara K; Ikeguchi M Ferritin Assembly Revisited: A Time-Resolved Small-Angle X-Ray Scattering Study. *Biochemistry* 2016, 55, 287–293. [PubMed: 26690025]
- (3). Ries J; Schwarze S; Johnson CM; Neuweiler H Microsecond Folding and Domain Motions of a Spider Silk Protein Structural Switch. *J. Am. Chem. Soc* 2014, 136, 17136–17144. [PubMed: 25382060]
- (4). Rimmerman D; Leshchev D; Hsu DJ; Hong J; Kosheleva I; Chen LX Direct Observation of Insulin Association Dynamics with Time-Resolved X-Ray Scattering. *J. Phys. Chem. Lett* 2017, 8, 4413–4418. [PubMed: 28853898]
- (5). Gaines WA; Sehorn MG; Marcotte WR Spidroin N-Terminal Domain Promotes a pH-Dependent Association of Silk Proteins during Self-Assembly. *J. Biol. Chem* 2010, 285, 40745–40753. [PubMed: 20959449]
- (6). Schwarze S; Zwettler FU; Johnson CM; Neuweiler H; Boles JO The N-Terminal Domains of Spider Silk Proteins Assemble Ultrafast and Protected from Charge Screening. *Nat. Commun* 2013, 4, 2815. [PubMed: 24240554]
- (7). Pelay-Gimeno M; Glas A; Koch O; Grossmann TN Structure-Based Design of Inhibitors of Protein-Protein Interactions: Mimicking Peptide Binding Epitopes. *Angew. Chem., Int. Ed. Engl* 54, 8896–8927. [PubMed: 26119925]
- (8). Nooren IMA; Thornton JM Structural Characterisation and Functional Significance of Transient Protein-Protein Interactions. *J. Mol. Biol* 2003, 325, 991–1018. [PubMed: 12527304]
- (9). Munoz V; Cerminara M When Fast Is Better: Protein Folding Fundamentals and Mechanisms from Ultrafast Approaches. *Biochem. J* 473, 2545–2559.
- (10). Akiyama S; Takahashi S; Kimura T; Ishimori K; Morishima I; Nishikawa Y; Fujisawa T Conformational Landscape of Cytochrome c Folding Studied by Microsecond-Resolved Small-Angle X-Ray Scattering. *Proc. Natl. Acad. Sci. U.S.A* 2002, 99, 1329–1334. [PubMed: 11773620]
- (11). Möller J; Léonardon J; Gorini J; Dattani R; Narayanan T A Sub-Ms Pressure Jump Setup for Time-Resolved X-Ray Scattering. *Rev. Sci. Instrum* 2016, 87, 125116. [PubMed: 28040915]
- (12). Kennis JTM; Groot M-L Ultrafast Spectroscopy of Biological Photoreceptors. *Curr. Opin. Struct. Biol* 2007, 17, 623–630. [PubMed: 17959372]
- (13). Ganim Z; Chung HS; Smith AW; DeFlores LP; Jones KC; Tokmakoff A Amide I Two-Dimensional Infrared Spectroscopy of Proteins. *Acc. Chem. Res* 2008, 41, 432–441. [PubMed: 18288813]
- (14). Sorigué D; Légeret B; Cuiné S; Blangy S; Moulin S; Billon E; Richaud P; Brugière S; Couté Y; Nurizzo D; et al. An Algal Photoenzyme Converts Fatty Acids to Hydrocarbons. *Science* 2017, 357, 903–907. [PubMed: 28860382]
- (15). Björn LO Photoactive Proteins In Photobiology; Springer: New York, NY, 2015; pp 139–150.
- (16). Cammarata M; Levantino M; Schotte F; Anfinrud PA; Ewald F; Choi J; Cupane A; Wulff M; Ihee H Tracking the Structural Dynamics of Proteins in Solution Using Time-Resolved Wide-Angle X-Ray Scattering. *Nat. Methods* 2008, 5, 881–886. [PubMed: 18806790]

- (17). Cai J; Yang J; Jones DP Mitochondrial Control of Apoptosis: The Role of Cytochrome c. *Biochim. Biophys. Acta, Bioenerg* 1998, 1366, 139–149.
- (18). Pascher T; Chesick JP; Winkler JR; Gray HB Protein Folding Triggered by Electron Transfer. *Science* 1996, 271, 1558–1560. [PubMed: 8599112]
- (19). Winkler JR Cytochrome c Folding Dynamics. *Curr. Opin. Chem. Biol* 2004, 8, 169–174. [PubMed: 15062778]
- (20). Telford JR; Tezcan FA; Gray HB; Winkler JR Role of Ligand Substitution in Ferrocycytochrome c Folding. *Biochemistry* 1999, 38, 1944–1949. [PubMed: 10026276]
- (21). Elove GA; Bhuyan AK; Roder H Kinetic Mechanism of Cytochrome c Folding: Involvement of the Heme and Its. *Biochemistry* 1994, 33, 6925–6935. [PubMed: 8204626]
- (22). Mara MW; Hadt RG; Reinhard ME; Kroll T; Lim H; Hartsock RW; Alonso-Mori R; Chollet M; Glowina JM; Nelson S; et al. Metalloprotein Entatic Control of Ligand-Metal Bonds Quantified by Ultrafast X-Ray Spectroscopy. *Science* 2017, 356, 1276–1280. [PubMed: 28642436]
- (23). Kroll T; Hadt RG; Wilson SA; Lundberg M; Yan JJ; Weng T-C; Sokaras D; Alonso-Mori R; Casa D; Upton MH; et al. Resonant Inelastic X-Ray Scattering on Ferrous and Ferric Bis-Imidazole Porphyrin and Cytochrome c : Nature and Role of the Axial Methionine–Fe Bond. *J. Am. Chem. Soc* 2014, 136, 18087–18099. [PubMed: 25475739]
- (24). Oellerich S; Wackerbarth H; Hildebrandt P Spectroscopic Characterization of Nonnative Conformational States of Cytochrome c. *J. Phys. Chem. B* 2002, 106, 6566–6580.
- (25). Colón W; Wakem LP; Sherman F; Roder H Identification of the Predominant Non-Native Histidine Ligand in Unfolded Cytochrome c. *Biochemistry* 1997, 36, 12535–12541. [PubMed: 9376358]
- (26). Takahashi S; Yeh S-R; Das TK; Chan C-K; Gottfried DS; Rousseau DL Folding of Cytochrome c Initiated by Submillisecond Mixing. *Nat. Struct. Mol. Biol* 1997, 4, 44–50.
- (27). Abbruzzetti S; Viappiani C; Small JR; Libertini LJ; Small EW Kinetics of Histidine Deligation from the Heme in GuHCl-Unfolded Fe(III) Cytochrome c Studied by a Laser-Induced pH-Jump Technique. *J. Am. Chem. Soc* 2001, 123, 6649–6653. [PubMed: 11439052]
- (28). Graber T; Anderson S; Brewer H; Chen Y-S; Cho HS; Dashdorj N; Henning RW; Kosheleva I; Macha G; Meron M; et al. BioCARS: A Synchrotron Resource for Time-Resolved X-Ray Science. *J. Synchrotron Radiat* 2011, 18, 658–670. [PubMed: 21685684]
- (29). Heinz B; Schmierer T; Laimgruber S; Gilch P Excited State Processes of Nitrobenzaldehydes Probed by Ultrafast Fluorescence and Absorption Spectroscopy. *J. Photochem. Photobiol., A* 2008, 199, 274–281.
- (30). Abbruzzetti S; Sottini S; Viappiani C; Corrie JET Acid-Induced Unfolding of Myoglobin Triggered by a Laser pH Jump Method. *Photochem. Photobiol. Sci* 2006, 5, 621–628. [PubMed: 16761091]
- (31). Donten ML; Hassan S; Popp A; Halter J; Hauser K; Hamm P pH-Jump Induced Leucine Zipper Folding beyond the Diffusion Limit. *J. Phys. Chem. B* 2015, 119, 1425–1432. [PubMed: 25536860]
- (32). Donten ML; Hamm P; VandeVondele J A Consistent Picture of the Proton Release Mechanism of O NBA in Water by Ultrafast Spectroscopy and Ab Initio Molecular Dynamics. *J. Phys. Chem. B* 2011, 115, 1075–1083. [PubMed: 21222460]
- (33). Donten ML; Hamm P pH-Jump Induced α -Helix Folding of Poly-L-Glutamic Acid. *Chem. Phys* 2013, 422, 124–130.
- (34). Oang KY; Yang C; Muniyappan S; Kim J; Ihée H SVD-Aided Pseudo Principal-Component Analysis: A New Method to Speed up and Improve Determination of the Optimum Kinetic Model from Time-Resolved Data. *Struct. Dyn* 2017, 4, 44013.
- (35). Schmidt M; Pahl R; Srajer V; Anderson S; Ren Z; Ihée H; Rajagopal S; Moffat K Protein Kinetics: Structures of Intermediates and Reaction Mechanism from Time-Resolved X-Ray Data. *Proc. Natl. Acad. Sci. U.S.A* 2004, 101, 4799–4804. [PubMed: 15041745]
- (36). Cho HS; Dashdorj N; Schotte F; Graber T; Henning R; Anfinrud P Protein Structural Dynamics in Solution Unveiled via 100-Ps Time-Resolved X-Ray Scattering. *Proc. Natl. Acad. Sci. U.S.A.* 2010, 107, 7281–7286. [PubMed: 20406909]

- (37). Kim KH; Oang KY; Kim J; Lee JH; Kim Y; Ihee H Direct Observation of Myoglobin Structural Dynamics from 100 Picoseconds to 1 Microsecond with Picosecond X-Ray Solution Scattering. *Chem. Commun* 2011, 47, 289–291.
- (38). Moffat K The Frontiers of Time-Resolved Macromolecular Crystallography: Movies and Chirped X-Ray Pulses. *Faraday Discuss.* 2003, 122, 65–77. [PubMed: 12555850]
- (39). Kim KH; Muniyappan S; Oang KY; Kim JG; Nozawa S; Sato T; Koshihara S-Y; Henning R; Kosheleva I; Ki H; et al. Direct Observation of Cooperative Protein Structural Dynamics of Homodimeric Hemoglobin from 100 Ps to 10 Ms with Pump-Probe X-Ray Solution Scattering. *J. Am. Chem. Soc* 2012, 134, 7001–7008. [PubMed: 22494177]
- (40). Cho HS; Schotte F; Dashdorj N; Kyndt J; Henning R; Anfinrud PA Picosecond Photobiology: Watching a Signaling Protein Function in Real Time via Time-Resolved Small- and Wide-Angle X-Ray Scattering. *J. Am. Chem. Soc* 2016, 138, 8815–8823. [PubMed: 27305463]
- (41). Choi J; Fujitsuka M; Tojo S; Majima T Folding Dynamics of Cytochrome c Using Pulse Radiolysis. *J. Am. Chem. Soc* 2012, 134, 13430–13435. [PubMed: 22812586]
- (42). Chen E; Goldbeck RA; Kliger DS Earliest Events in Protein Folding: Submicrosecond Secondary Structure Formation in Reduced Cytochrome c. *J. Phys. Chem. A* 2003, 107, 8149–8155.
- (43). Telford JR; Wittung-Stafshede P; Gray HB; Winkler JR Protein Folding Triggered by Electron Transfer. *Acc. Chem. Res* 1998, 31, 755–763.
- (44). Kikhney AG; Svergun DI A Practical Guide to Small Angle X-Ray Scattering (SAXS) of Flexible and Intrinsically Disordered Proteins. *FEBS Lett* 2015, 589, 2570–2577. [PubMed: 26320411]
- (45). Li W; Wang J; Zhang J; Wang W Molecular Simulations of Metal-Coupled Protein Folding. *Curr. Opin. Struct. Biol* 2015, 30, 25–31. [PubMed: 25523438]

Figure 1.
Scheme of cyt *c* heme iron axial ligation dynamics as previously revealed by OTA studies.

Figure 2.

X-ray solution scattering data for cyt *c* pH-jump experiment. (a) TRXSS signals recorded for cyt *c* and O-NBA mixture and pure O-NBA solution 20 ms after the laser excitation. The heating signal observed in pure O-NBA measurements was used for subtracting the solvent contribution and extracting the protein-related signal as explained in the text. (b) Comparison of the protein signal at 20 ms with the difference signal produced by subtracting static scattering curves measured at pH values of 4.0 and 5.1. (c) Time series of the protein-associated TRXSS signals obtained after subtraction of the solvent contribution. (d) Population dynamics of the observed species I_H^+ and U_H^+ obtained from the global analysis and T . (e) Species-associated scattering difference curves obtained from the global analysis.

Figure 3.

X-ray solution scattering data for cyt *c* photoreduction experiment. (a) TRXSS signals recorded at 500 μs after photoreduction of heme iron in cyt *c* by $[\text{Ru}(\text{bpy})_3]^{2+}$ in solution with pH values of 4 and 7. (b) Time series of the protein-associated TRXSS signals recorded at pH = 4. The inset shows the time dependence of the absolute scattering difference signal integrated at $q < 0.05 \text{\AA}^{-1}$.