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Fluorescent iron-sulfur centers: photochemistry of the PetA Rieske protein from *Aquifex aeolicus*

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Abstract

Cytochrome bc_1 complexes are energy-transducing enzymes and key components of respiratory electron chains. They contain Rieske 2Fe-2S proteins that absorb very weakly in the visible absorption region compared to the heme cofactors of the cytochromes, but are known to yield photoproducts. Here, the photoreactions of isolated Rieske proteins from the hyperthermophilic bacterium *Aquifex aeolicus* are studied in two redox states using ultrafast transient fluorescence and absorption spectroscopy. We provide evidence, for the first time in iron-sulfur proteins, of very weak fluorescence of the excited state, in the oxidized as well as the reduced state. The excited states of the oxidized and reduced forms decay in 1.5 ps and 30 picoseconds, respectively. In both cases they give rise to product states with lifetimes beyond 1 nanosecond, reflecting photo-reduction of oxidized centers as well as photo-oxidation of reduced centers. Potential reaction partners are discussed and studied using site-directed mutagenesis. For the reduced state, a nearby disulfide bridge is suggested as an electron acceptor. The resulting photoproducts in either state may play a role in photoactivation processes.

Keywords: Rieske protein, 2Fe-2S center, fluorescence, ultrafast spectroscopy, electron transfer

1. Introduction

Functional and non-functional photochemical effects in colored protein systems are predominantly studied in proteins binding large ring-containing cofactors such as chlorophyll, carotene, flavin, coumarin, heme and retinal. Yet, metal-atom cofactor containing proteins may also absorb visible light, albeit with relatively low extinction [1]. These include iron-sulfur (Fe-S) proteins that bind iron atoms cross-linked with cysteine residues via their sulfur atoms [2], and that weakly absorb throughout the visible spectrum, especially in the blue spectral region [3], through bands assigned to ligand-to-metal charge transfer transitions [4]. Fe-S proteins are mostly involved as intermediates in biochemical electron-transfer chains as they can readily adopt different redox states. Spectroscopically, EPR is often the method of choice to study these redox processes, as the optical signals are small and easily overwhelmed by signals of more strongly absorbing redox partners.

Whereas the functioning of Fe-S centers, and of Fe-containing non-heme metalloproteins in general, is not thought to involve light absorption, there have been early indications of photoactivity (in particular leading to singlet oxygen generation) induced by absorption of photons by Fe-S clusters in native respiratory complexes and artificial proteins [5, 6]. The initial processes following the absorption of light by such systems have been investigated to some extent only in very recent years. In 2017, as an unexpected side result of a transient absorption study on the photochemistry of hemes in a bacterial cytochrome *bc*₁ complex that takes place on the timescale of a few picoseconds, we reported a weak signal lasting much longer and occurring upon excitation in the blue spectral region [7]. This signal was assigned to photo-oxidation of reduced [2Fe-2S] centers. Subsequently, in the same year, a more extensive transient absorption study of an oxidized [2Fe-2S] center in a bacterial

ferredoxin was reported by Larsen and coworkers [8]. Here, states with lifetimes in the nanosecond range were assigned to photo-reduction products populated from excited states decaying within a few picoseconds. These studies were then extended to different types of Fe-S centers [9]. On the other hand, very recently a reaction intermediate in pyruvate formate-lyase activating enzyme observed by EPR after prolonged illumination was suggested to be initiated by electron transfer from the reduced [4Fe-4S] center [10]. Further, in nitrogenase photolysis of Fe-S bonds at cryogenic temperatures following electron transfer has also been proposed from nanosecond time-resolved EPR experiments [11] and photolysis of [2Fe-2S]-NO bonds has been observed [12]. Altogether, photoproducts from both oxidized and reduced Fe-S centers are emerging as precursors for photoactivation processes in proteins. Yet, the initially formed excited states and photoproducts remain poorly characterized.

In the present work, we investigate the excited state and product state dynamics on the femtosecond to nanosecond timescale of a Rieske [2Fe-2S] protein [13, 14]. This choice was inspired by our previous work on the respiratory cytochrome bc_1 complex from the purple bacterium *Rhodobacter capsulatus* [7]. Rieske proteins are essential constituents of cytochrome bc_1 complexes, with their [2Fe-2S] cluster acting as an electron transfer intermediate between external quinones and the c_1 heme. The work in this paper concerns the isolated soluble form of one of the two Rieske proteins from the hyperthermophilic bacterium *Aquifex aeolicus*, PetA. This protein has been well characterized and is relatively stable in the isolated form [15], and other colored cofactors are absent. Very recently its structure within the bc_1 complex was determined using cryo-electron microscopy [16]. In the Rieske [2Fe-2S] cluster, the two iron atoms are bridged by sulfur atoms, one iron atom being further coordinated by two more cysteine sulfur atoms, the other by two histidine nitrogen atoms [2]. The latter atom, more surface-exposed, is thought to be the redox-active site [13]. The ligation of an Fe atom to

histidine residues is specific for Rieske proteins; in other Fe-S proteins quasi-exclusively Fe-cysteine-ligation occurs [2, 13].

This study presents comprehensive spectroscopic data on the initial light-induced processes in a Rieske [2Fe-2S] protein. Our experiments concern both the oxidized (Fe-III, Fe-III) and the reduced (Fe-III, Fe-II) state of the protein, and are performed with transient absorption spectroscopy, as the previous studies, as well as transient fluorescence. The latter approach allowed us to report the first, very weak, fluorescence signals from Fe-S proteins, and directly correlate them with excited states. Our study represents the first characterization of the photochemistry of an Fe-S protein in its two physiologically relevant redox states and shows that in the same protein photoreduction and photooxidation of Fe-S centers can take place.

2. Materials and Methods

A truncated form of the PetA Rieske protein from *A. aeolicus* (strain VF5; UniprotKB O66460) without its transmembrane N-terminal domain (residues 1 to 42) was heterologously expressed from a pQE80L plasmid in *E. coli* BL21DE3. The 6xHis-tagged proteins were purified from cell-free extracts by gravity-flow chromatography on Ni-TED columns (Protino Ni-TED, Macherey-Nagel), followed by imidazole removal on Econo-Pac 10DG columns (BIO-RAD). Purified proteins were suspended in 50 mM Tris buffer, pH 8.0 containing 150 mM NaCl and all experiments were performed in this buffer, at a concentration of ~250-600 μM in [2Fe-2S] centers, based on an extinction coefficient at 450 nm of $5800 \text{ M}^{-1}\text{cm}^{-1}$ for the oxidized form, as established for the *Thermus thermophilus* Rieske protein [17]. The purified protein was in the reduced form; the oxidized form was obtained by addition of potassium ferricyanide.

All experiments were performed in 1-mm optical pathlength cells. Steady-state spectra were obtained using a Shimadzu UV-Vis 1700 spectrometer. Ultrafast transient absorption experiments using a white-light continuum probe pulse, and transient fluorescence experiments using a Kerr-gate setup [18] with CS₂ as a Kerr medium (full width half maximum of the temporal response function ~1 ps), were performed as in ref. [19], using the short-lived dye NK88 as a reference. Both types of experiments were performed with a 390 nm pump pulse and at 500 Hz repetition rate. The intensity of the pulses was set to ~700 nJ and the waist of the beam at the level of the sample ~100-120 μm; we estimate that 15-25% of the centers in the excited volume absorbed a photon at each shot.

For all time-resolved experiments the sample was thermostatted at 10 °C. Global data analysis of the spectro-temporal data sets was performed using Glotaran [20].

3. Results

3.1 Steady-state absorption spectra.

Fig. 1 shows absorption spectra of the protein as prepared and after chemical oxidation by a stoichiometric amount of ferricyanide. The spectra are very similar to those reported for the reduced and oxidized bovine [3], *Sulfolobus solfataricus* [21] and *T. thermophilus* [17] Rieske proteins. From the effect of dithionite addition under anaerobic conditions we estimate the as-prepared protein was 80-90% reduced, somewhat depending on the preparation. As in the presence of dithionite the Fe-S centers were relatively unstable, ultrafast time-resolved experiments were performed on the as-prepared proteins with ~90% reduced proteins (denoted as ‘reduced’) and ferricyanide-treated (oxidized) proteins. The experiments are performed

below the pK of the histidines both for the oxidized and reduced state of the Fe-S center [22], so the redox change does not involve a large change in protonation state.

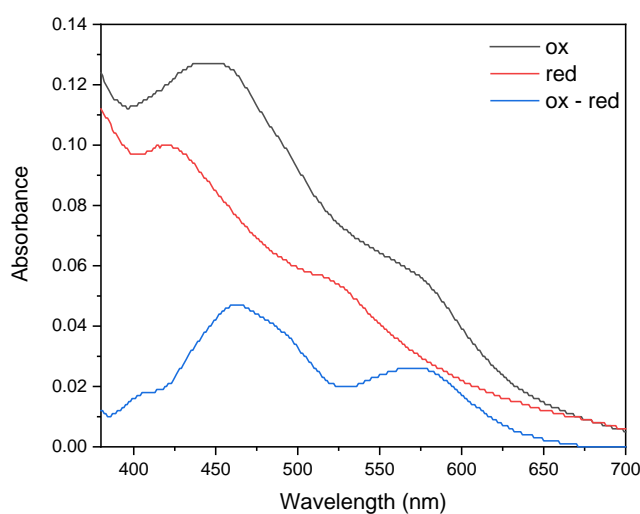


Fig. 1. Absorption spectra of PetA as prepared (reduced form) and after addition of ferricyanide (oxidized form) until the spectrum > 480 nm (where ferricyanide does not absorb) remained unchanged. The calculated difference spectrum is also shown.

3.2 Time-resolved fluorescence.

Upon excitation at 390 nm, the oxidized protein displays very weak, but measurable, fluorescence. Fig. 2A shows fluorescence spectra of the oxidized protein at different delay times. The large signal around 460 nm in the spectrum at $t = 0.8$ ps (in the tail of the temporal response of the instrument) is due to the (instantaneous) Raman signal of water. The spectra decay in a few picoseconds. Fig. 2B shows the kinetics at 485 nm, where the Raman signal has no contribution. (Fig. S1 shows the kinetics at 475 nm, where both the Raman signal and the fluorescence decay contribute). The decay clearly extends for a few picoseconds beyond the temporal instrument response (Fig. 2B) and can be described with a time constant of ~ 1.5 ps; this time constant was fixed based on transient absorption experiments (see below). A global fit of the data after the instrument response function (where the Raman signal contributes at the blue side) shows that the spectrum associated with the fluorescence decay is broad and peaks around 470 nm (Fig. 2E).

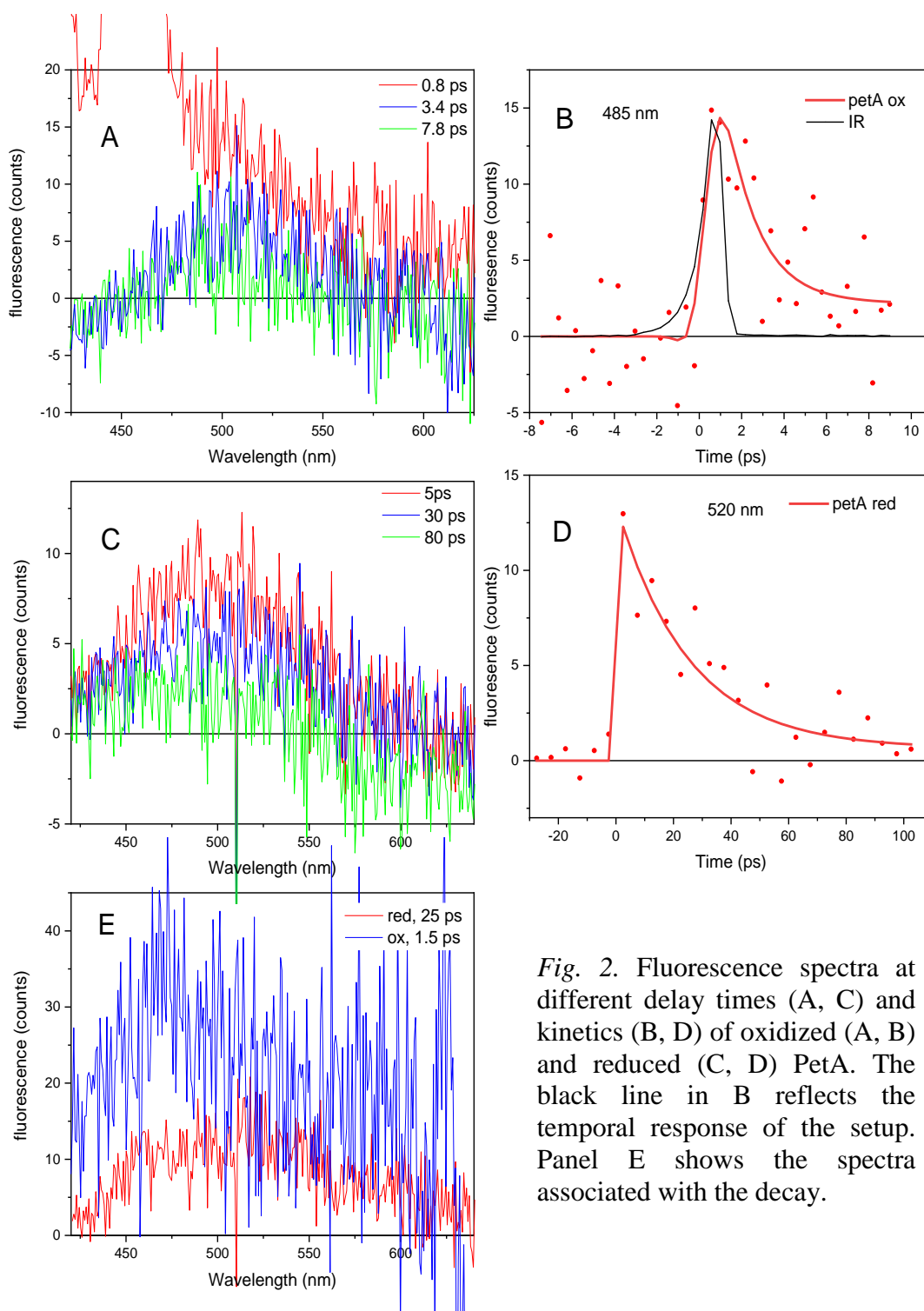


Fig. 2. Fluorescence spectra at different delay times (A, C) and kinetics (B, D) of oxidized (A, B) and reduced (C, D) PetA. The black line in B reflects the temporal response of the setup. Panel E shows the spectra associated with the decay.

In the reduced protein, weak emission is also observed (Fig. 2C). It decays on a longer time scale (~ 25 ps) than that of the oxidized form (Fig. 2D). The maximum of the spectrum associated with this decay is situated more to the red.

In both cases, a very small long-lived component remains that is also observed in the absence of the protein in the sample cell; this background pump-probe signal of the Kerr-gate setup [18] is not considered here.

The intensity of the fluorescence per absorbed photon extrapolated to $t=0$ (i.e. that corresponding to the decay associated spectra of Fig. 2E) is 2-3 orders of magnitude lower than that of cyanine dyes like NK88. This comparison indicates that the intrinsic radiative decay rate (the probability of returning to the ground state by photon emission per unit of time) of the initially populated excited state is very low.

3.3 Time-resolved absorption.

Time-resolved absorption experiments were performed with pump pulses polarized at magic angle (54.7°) with respect to the white light probe pulses in order to avoid photoselection effects.

For the oxidized protein, significant absorption changes were observed throughout the visible spectrum. Fig. 3A shows the kinetics at selected wavelengths. Initial bleaching is observed around 460 nm, near the maximum of the ground state absorption, and induced absorption on the blue and red side of the spectrum. This signal evolves within a few picoseconds to a spectrum with negative features at 460 and 580 nm, and induced absorption between these features and on the blue and red sides of the spectrum. This spectrum remains unchanged on the 1-ns timescale. Any evolution on the timescale of a few hundred femtoseconds is masked by relatively strong cross-phase modulation artifacts during the pump-probe temporal overlap. Fig. 3B shows the results of a global analysis in terms of a 1.5-ps phase and a long-lived phase. The shape of the initial spectrum roughly follows that of the inverted

absorption spectrum, as expected for light-induced depletion of the ground-state, and is also composed of a broad induced absorption. The long-lived phase has more pronounced features that appear to correlate with the steady-state reduced-minus-oxidized spectrum, suggesting that the state corresponds to a reduced Fe-S center. Comparison of the amplitude of the modulations in the steady-state and long-live phase difference spectra indicates a photo-reduction yield in the order of 40-70% per absorbed photon.

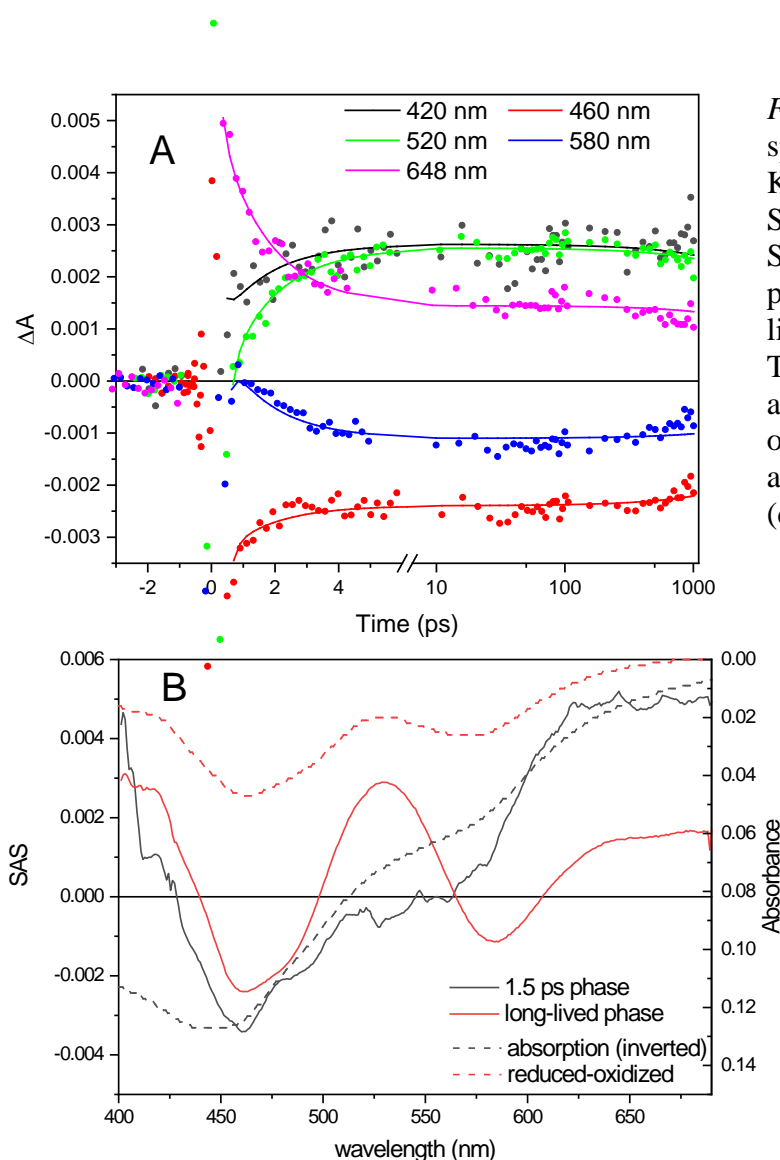


Fig. 3. Transient absorption spectroscopy of oxidized PetA. A. Kinetics at selected wavelengths. Solid lines are from a global fit. B. Species-associated spectra of the precursors of the 1.5 ps and long-lived phase (solid lines, left axis). The inverted ground-state absorption spectrum and the red-ox spectrum (taken from Fig. 1) are plotted for comparison (dashed lines, right axis).

To investigate possible electron donors, in Fig. 4 the long-lived spectral phase is compared with model spectra. Scaling the long-lived phase to the steady-state red-ox spectrum so that their difference is roughly flat at $\lambda > 600$ nm (where cation amino acid radicals are not

expected to absorb, see below) yields a broad induced absorption, which is superimposed on a positive feature at ~ 530 nm. As no other cofactors are present in PetA, intrinsic protein constituents are potential electron donors, in particular tryptophan and tyrosine aromatic residues [23]. Fig. 4 also shows model spectra of Trp radical species that absorb in the visible [24]. The Tyr cation radical ($\text{TyrOH}^{\bullet+}$) has recently also been shown to absorb around 490 nm, but this species is thought to be highly unstable [19, 25]. $\text{TrpH}^{\bullet+}$ is a potential initial cation product state, but its spectrum, with a broad maximum around 580 nm is much red-shifted with respect to the positive feature observed around 530 nm. However the spectrum of the deprotonated radical Trp^{\bullet} is close to this feature (Fig. 4) and accordingly the constructed ($\text{Fe-S}_{\text{red}} \text{Trp}^{\bullet}$) minus (Fe-S_{ox}) spectrum (neutral Trp does not absorb in the visible) is reminiscent of the spectrum of the long-lived phase. This analysis thus would suggest that the photoreaction of oxidized PetA may involve electron abstraction by the excited Fe-S center from a nearby Trp associated with very rapid (<1.5 ps) deprotonation of the Trp radical.

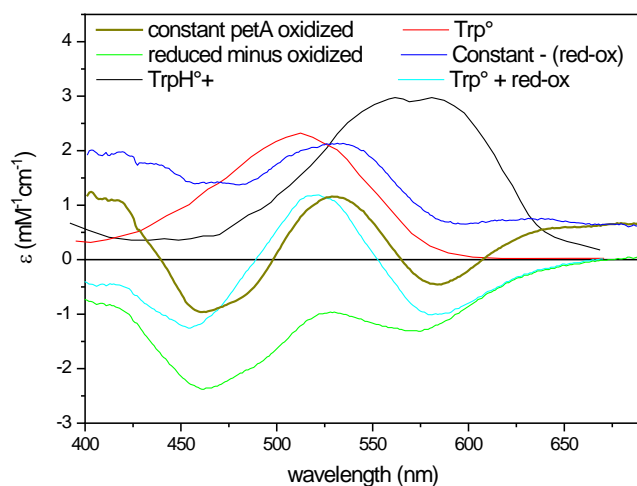


Fig. 4. Comparison of the long-lived phase observed in transient absorption of oxidized PetA (from Fig. 3B) with model spectra. The long-lived phase is scaled with respect to the steady-state red-ox spectrum (in extinction units) so that their difference is roughly flat at $\lambda > 600$ nm. The tryptophan radical model spectra are taken from ref. 24

Although the two Trp residues (Trp 62 and Trp 120) that the Rieske protein harbors are located relatively far from the Fe-S center (see Discussion), the possible involvement of these residues was investigated by studying genetically modified proteins where these residues were changed to redox-inactive phenylalanines. Yet, the kinetics of excited state decay, as well as

the final product state spectrum of the W62F and W120F variants (Fig. 5), were found to be very similar to those of the wild type protein (Fig. 3), implying that these Trp residues are not involved in the photochemical processes.

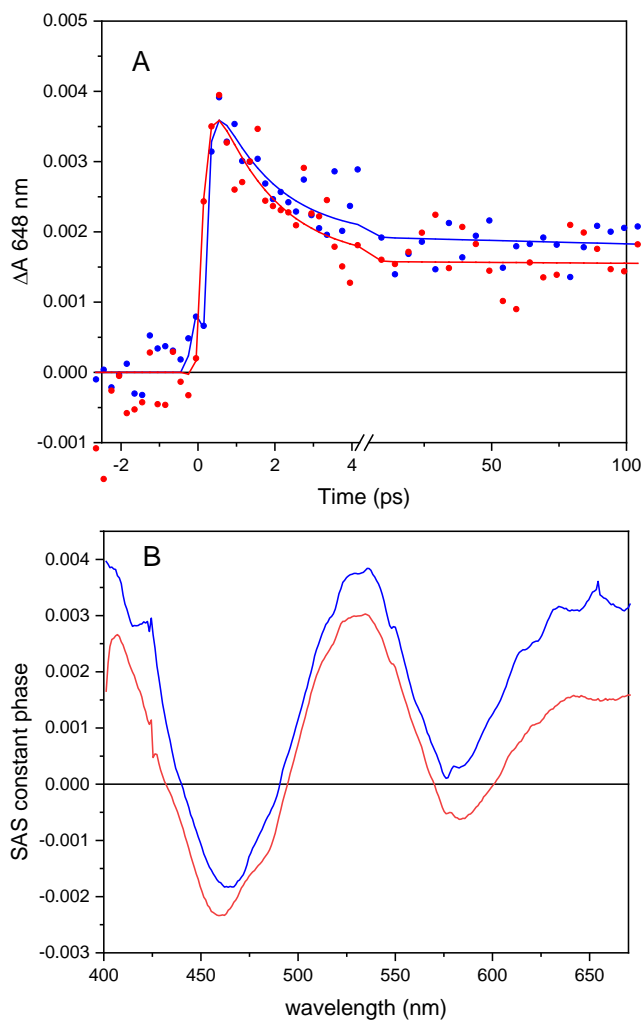


Fig. 5. Transient absorption spectroscopy of the oxidized form of W62F (blue) and W120F (red) PetA. A. The kinetics at 648 nm (normalized), B. long-lived asymptotic spectra from the global analysis.

For the reduced protein, also sizeable transient absorption signals were observed (Fig. 6), although these were substantially smaller than for the oxidized protein. A spectral evolution, with maximal amplitude around 450 nm, was observed with a time constant of ~ 30 ps. The bleaching minima in the initial and final difference spectra correspond with the absorption features in the ground-state spectra (Fig. 6B), yet the transient spectra are more pronounced. The minima and maxima in both the initial and the long-lived spectrum resemble those of the steady-state oxidized minus reduced spectrum, indicating that photo-oxidation occurs.

Comparison of the amplitude of the modulations in the steady-state and long-live phase difference spectra indicates a photo-oxidation yield in the order of 10-17% per absorbed photon. The spectrum associated with the long-lived photoproduct is predominantly negative, whereas the steady-state oxidized minus reduced spectrum is all-positive. This implies that only part of the corresponding product state population concerns oxidized Fe-S centers.

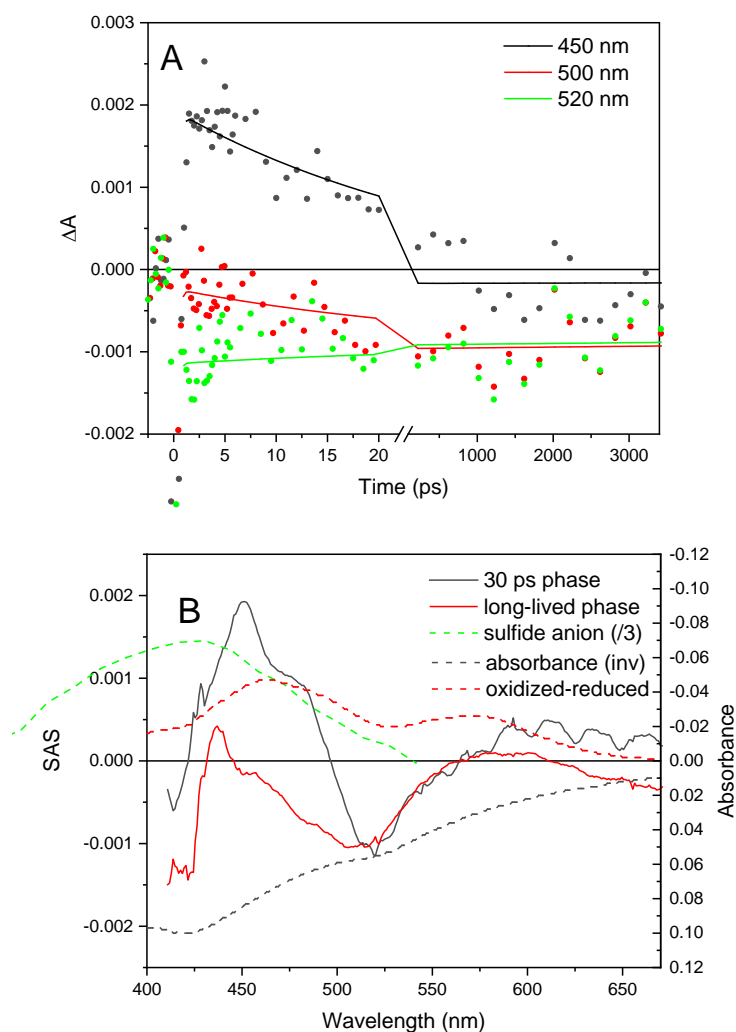


Fig. 6. Transient absorption spectroscopy of reduced PetA. A. Kinetics at selected wavelengths. Solid lines are from a global fit. B. Species-associated spectra of the precursors of the 30 ps and long-lived phase (solid lines, left axis). The redox difference spectrum (red dashed), the inverted ground-state absorbance spectrum (dashed line, right axis) (both taken from Fig. 1) and a cystine sulfide anion spectrum from ref. 34 are plotted for comparison.

4. Discussion

Following precursory indications of involvement of long-lived photoproducts assignable to the Rieske 2Fe-2S protein moiety of the intact bc_1 complex of a photosynthetic

bacterium [7], we have now presented comprehensive spectroscopic data on the initial light-induced processes in a Rieske 2Fe-2S protein itself. Isolated Rieske proteins are notoriously unstable, but soluble PetA from the hyperthermophilic bacterium *A. aeolicus* could be prepared and maintained in both the oxidized and reduced form for sufficiently long times to allow detailed spectroscopic investigations. This allowed the first characterization of the photochemistry of an Fe-S protein in its two physiologically relevant redox states.

A remarkable result is the observation of fluorescence for both redox states. To the best of our knowledge the data presented in Fig. 2 constitutes the first evidence of fluorescence from any Fe-S protein so far. The emissions are extremely weak and short-lived and therefore require both temporal gating and long signal averaging to be detectable above the background. The amplitudes of the fluorescence are more than two orders of magnitude below that of organic dyes; correspondingly we estimate the intrinsic radiative lifetime on the order of hundreds of nanoseconds or more. The observed lifetimes are much shorter (~ 1.5 ps for Fe-S_{ox}, ~ 25 ps for Fe-S_{red}), an observation that we assign to quenching by charge transfer reactions. Indeed, the observed fluorescence lifetimes correspond well with the time constants observed in the transient absorption experiments, for both redox states. We note that in a study on an oxidized ferredoxin 2Fe-2S system, Mao and coworkers assigned sharp negative absorption features around 460 and 480 nm associated with phases on the femtosecond to nanosecond timescale partly to stimulated emission [8], the transient absorption counterpart of fluorescence. Our transient absorption results of the oxidized Rieske protein show substantial similarity with those data, but our assessment of the broad spectrum and the low yield of the fluorescence from independent transient fluorescence measurements indicate that the feature is more likely associated with ground-state bleach.

The fluorescence spectra observed for both the oxidized and the reduced forms upon excitation at 390 nm are remarkably broad (Fig. 2E), spanning nearly completely the visible

range and in particular largely overlapping the red part of the absorption spectrum. Theoretical studies indicate that a distribution of numerous transitions underlies the broad absorption bands of 2Fe-2S clusters [4, 8, 26]. Our results thus imply that the initially excited states do not fully relax to the lowest excited states on the picosecond timescale of the quenching of the fluorescence by charge transfer reactions.

Intriguingly, the transient absorption spectra associated with the long-lived phases for the oxidized and the reduced proteins display roughly inverted maximum and minimum features at ~450, 525 and 580 nm (Figs. 3B, 6B). Comparison with the steady-state redox different spectrum indicates that they correspond to reduction and oxidation of the Fe-S centers respectively. Indeed, in previous studies based on similar resemblances between steady-state and transient spectra in the visible range, photoreduction of Fe-S_{ox} has been inferred for a number of Fe-S proteins [8, 9]. Also, we have previously suggested photo-oxidation of the Rieske 2Fe-2S cluster in the *Rhodobacter capsulatus bc₁* complex, but this assessment was complicated by the dominant absorption of the hemes in this complex [7]. Altogether, our present work demonstrates such photo-oxidation and moreover shows that in the same protein photoreduction and photooxidation of Fe-S centers can take place.

The spectrum associated with the long-lived photoproduct of the (Fe-S)_{ox} state of the Rieske protein (Fig. 3B) is similar to those observed on the picosecond-nanosecond timescale in other oxidized 2Fe-2S proteins [8, 9] and comprises both positive and negative (440-500 nm and around 580 nm) absorption changes. As indicated before, the similarity of the extrema with those of the steady-state reduced-minus-oxidized spectrum (Fig. 3B, 4) is strongly indicative of photo-reduction of the Fe-S center. Yet, throughout the visible range steady-state (Fe-S)_{red} displays a substantially lower extinction than (Fe-S)_{ox}; this holds true for the Rieske protein (Fig. 1, Refs. [3, 17, 21]), as well as for solutions of other 2Fe-2S proteins [27-29]. Therefore

the photoproduct appears to include also induced absorption from other species, either from a protein-derived electron donor or from the 2Fe-2S center itself.

Our spectral analysis (Fig. 4) suggested a possible involvement of a tryptophan radical. Formation of a $(\text{Fe-S})_{\text{red}}\text{TrpH}^{\circ+}$ radical pair from the excited state $(\text{Fe-S})_{\text{ox}}^*\text{TrpH}$ state is energetically feasible: given the midpoint potentials of the $(\text{Fe-S})_{\text{ox}}/(\text{Fe-S})_{\text{red}}$ (0.21 V [15]) and $\text{TrpH}^{\circ+}/\text{TrpH}$ (1.15 V [30]) redox couples, this state lies 0.94 eV above the ground-state, much lower than the 2.6 eV corresponding to the fluorescence maximum (~ 470 nm, Fig. 2C) from the $(\text{Fe-S})_{\text{ox}}^*$ state. The *A. aeolicus* PetA protein harbors two tryptophan residues [31], Trp 62 and Trp 120 (numbering *A. aeolicus*). Trp 62 is conserved in the proteobacteria *Chromatium vinosum*, *Helicobacter pylori*, *Paracoccus denitrificans* and *Thiobacillus ferrooxidans*, as well as in the bovine Rieske protein [31], whereas Trp 120 does not appear to be widely conserved. In *A. aeolicus* these Trp residues are located ~ 24 Å and ~ 15 Å from the Fe-S center [16]. These distances appear too high for allowing direct electron transfer on the picosecond timescale, and indeed we found that mutant proteins where these residues were replaced by redox-inactive phenylalanines behaved similar as the WT protein (Fig. 5). By elimination, we suggest that the photoreduction of the Fe-S center is associated with an additional modification of its electronic structure leading to enhanced absorption in the visible range.

Finally, our results on the $(\text{Fe-S})_{\text{red}}$ state constitute the first evidence for photo-oxidation of a reduced Fe-S center. Intriguingly, the transient absorption spectra associated with both the initial excited state and the final product state carry $(\text{Fe-S})_{\text{ox}}$ *minus* $(\text{Fe-S})_{\text{red}}$ signatures, with extrema at ~ 450 , 525 and 580 nm. This suggests that some charge separation takes place already concomitant with formation of the initial excited state and that the excited state very rapidly (sub-picosecond) equilibrates with a charge-separated state. Unfortunately, with our setup fluorescence decays occurring on the femtosecond timescale are difficult to resolve in the Rieske protein due to the combination of extremely low fluorescence yield and low efficiency

of the Kerr gate medium required for the corresponding temporal resolution [18]. In searching nearby potential electron acceptors, an interesting candidate is the disulfide bridge between Cys116 and Cys137 that is located close to the redox-active Fe atom of the Fe-S center (it is at 3.6 Å from the Cys116 sulfur atom [16]) and stabilizes the Rieske center [32, 33]. The sulfide radical anion has a sizeable absorption below 500 nm with a maximum near 420 nm [34] that roughly corresponds to the positive features (420-500 nm) in the transient spectrum (Fig. 6B). In this tentative interpretation, the excitation of the reduced (Fe-S) center thus leads to rupture of the disulfide bond. We emphasize that not only charge transfer products, but also strictly excited state spectral features and possibly other Fe-S center transformations (see below) are expected to contribute to the spectrum associated with the 30-ps phase.

The final state observed after the ~25-ps decay of the excited state also clearly corresponds to oxidation of the Fe-S center, albeit that the yield of this photo-oxidation is substantially lower than that of photo-reduction of the oxidized protein. The final absorbance difference spectrum has largely a negative amplitude (Fig. 6B, in particular between 450 and 550 nm), whereas the steady-state (Fe-S)_{ox} *minus* (Fe-S)_{red} spectrum (and the absorption of the sulfide anion radical tentative photoproduct) is all-positive. This indicates that this state also corresponds to a transformation of the (Fe-S)_{red} state other than its oxidation. We suggest there is partial photodissociation of the Fe-S center. This possibility will be further investigated in future work.

In conclusion, we have demonstrated that iron sulfur centers can fluoresce in the visible domain. The decay of the emitting excited state is associated with electron transfer to or from the center depending on the initial redox state. The resulting photoproducts in the Rieske protein as well as in other iron sulfur proteins may play a role as precursors of photoactivation processes. Extension of these studies to a longer timescale are required to assess the fate and stability of the photoproducts highlighted in this work.

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References

- [1] R.H. Holm, P. Kennepohl, E.I. Solomon, Structural and Functional Aspects of Metal Sites in Biology, *Chem. Rev.*, 96 (1996) 2239-2314.
- [2] J. Meyer, Iron–sulfur protein folds, iron–sulfur chemistry, and evolution, *J. Biol. Inorg. Chem.*, 13 (2008) 157-170.
- [3] J.S. Rieske, D.H. MacLennan, R. Coleman, Isolation and properties of an iron-protein from the (reduced coenzyme Q)-cytochrome *C* reductase complex of the respiratory chain, *Biochem. Biophys. Res. Comm.*, 15 (1964) 338-344.
- [4] L. Noodleman, E.J. Baerends, Electronic structure, magnetic properties, ESR, and optical spectra for 2-iron ferredoxin models by LCAO- $X\alpha$ valence bond theory, *J. Am. Chem. Soc.*, 106 (1984) 2316-2327.
- [5] C.S. Kim, J. Jung, Iron-Sulfur Centers as Endogenous Blue Light Sensitizers in Cells: a Study with an Artificial Non-Heme Iron Protein *Photochem. Photobiol.*, 56 (1992) 63-68.
- [6] K. Chang Sook, J. Jung, Inactivation of the respiratory chain in plant mitochondria by visible light: the primary target for photodamage and endogenous photosensitizing chromophores, *J. Photochem. Photobiol. B*, 29 (1995) 135-139.
- [7] M.H. Vos, B.J. Reeder, F. Daldal, U. Liebl, Ultrafast photochemistry of the *bc₁* complex, *Phys. Chem. Chem. Phys.*, 19 (2017) 6807-6813.
- [8] Z. Mao, E.C. Carroll, P.W. Kim, S.P. Cramer, D.S. Larsen, Ultrafast Charge-Transfer Dynamics in the Iron–Sulfur Complex of *Rhodobacter capsulatus* Ferredoxin VI, *J. Phys. Chem. Lett.*, 8 (2017) 4498-4503.
- [9] Z. Mao, S.-H. Liou, N. Khadka, F.E. Jenney, D.B. Goodin, L.C. Seefeldt, M.W.W. Adams, S.P. Cramer, D.S. Larsen, Cluster-Dependent Charge-Transfer Dynamics in Iron–Sulfur Proteins, *Biochemistry*, 57 (2018) 978-990.
- [10] H. Yang, E.C. McDaniel, S. Impano, A.S. Byer, R.J. Jodts, K. Yokoyama, W.E. Broderick, J.B. Broderick, B.M. Hoffman, The Elusive 5' -Deoxyadenosyl Radical: Captured and Characterized by Electron Paramagnetic Resonance and Electron Nuclear Double Resonance Spectroscopies, *J. Am. Chem. Soc.*, 141 (2019) 12139-12146.
- [11] D.A. Lukyanov, M.D. Krzyaniak, D.R. Dean, M.R. Wasielewski, L.C. Seefeldt, B.M. Hoffman, Time-Resolved EPR Study of H₂ Reductive Elimination from the Photoexcited Nitrogenase Janus E4(4H) Intermediate, *J. Phys. Chem. B*, 123 (2019) 8823-8828.

- [12] Y. Wang, J. Lee, H. Ding, Light-induced release of nitric oxide from the nitric oxide-bound CDGSH-type [2Fe–2S] clusters in mitochondrial protein Miner2, *Nitric Oxide*, 89 (2019) 96-103.
- [13] D.J. Ferraro, L. Gakhar, S. Ramaswamy, Rieske business: Structure–function of Rieske non-heme oxygenases, *Biochem. Biophys. Res. Comm.*, 338 (2005) 175-190.
- [14] T.A. Link, The Structures of Rieske and Rieske-Type Proteins, in: A.G. Sykes (Ed.) *Advances in Inorganic Chemistry*, Academic Press, Place Published, 1999, pp. 83-157.
- [15] M. Schütz, B. Schoepp-Cothenet, E. Lojou, M. Woodstra, D. Lexa, P. Tron, A. Dolla, M.-C. Durand, K.O. Stetter, F. Baymann, The Naphthoquinol Oxidizing Cytochrome *bc₁* Complex of the Hyperthermophilic Knallgasbacterium *Aquifex aeolicus*: Properties and Phylogenetic Relationships, *Biochemistry*, 42 (2003) 10800-10808.
- [16] G. Zhu, H. Zeng, S. Zhang, J. Juli, X. Pang, J. Hoffmann, Y. Zhang, N. Morgner, Y. Zhu, G. Peng, H. Michel, F. Sun, A 3.3 Å-Resolution Structure of Hyperthermophilic Respiratory Complex III Reveals the Mechanism of Its Thermal Stability, *Angew. Chem. Int. Ed.*, 59 (2020) 343-351.
- [17] J.A. Fee, K.L. Findling, T. Yoshida, R. Hille, G.E. Tarr, D.O. Hearshen, W.R. Dunham, E.P. Day, T.A. Kent, E. Münck, Purification and characterization of the Rieske iron-sulfur protein from *Thermus thermophilus*. Evidence for a [2Fe-2S] cluster having non-cysteine ligands, *J. Biol. Chem.*, 259 (1984) 124-133.
- [18] S.P. Laptanok, P. Nuernberger, A. Lukacs, M.H. Vos, Subpicosecond Kerr-gate spectrofluorometry, in: Y. Engelborghs, A.J.W.G. Visser (Eds.) *Methods in Molecular Biology, Fluorescence Spectroscopy and Microscopy: Methods and Protocols*, Vol. 1076, Humana Press, Place Published, 2014, pp. 321-336.
- [19] L. Nag, P. Sournia, H. Myllykallio, U. Liebl, M.H. Vos, Identification of the TyrOH^{•+} Radical Cation in the Flavoenzyme TrmFO, *J. Am. Chem. Soc.*, 139 (2017) 11500-11505.
- [20] J.J. Snellenburg, S.P. Laptanok, R. Seger, K.M. Mullen, I.H.M. van Stokkum, Glotaran: A Java-Based Graphical User Interface for the R Package TIMP, *J. Stat. Software*, 49 (2012).
- [21] T. Iwasaki, A. Kounosu, Y. Tao, Z. Li, J.E. Shokes, N.J. Cospers, T. Imai, A. Urushiyama, R.A. Scott, Rational Design of a Mononuclear Metal Site into the Archaeal Rieske-type Protein Scaffold, *J. Biol. Chem.*, 280 (2005) 9129-9134.
- [22] Y. Zu, M.M.J. Couture, D.R.J. Kolling, A.R. Crofts, L.D. Eltis, J.A. Fee, J. Hirst, Reduction Potentials of Rieske Clusters: Importance of the Coupling between Oxidation State and Histidine Protonation State, *Biochemistry*, 42 (2003) 12400-12408.
- [23] J.R. Winkler, H.B. Gray, Could tyrosine and tryptophan serve multiple roles in biological redox processes?, *Phil. Trans. A*, 373 (2014).
- [24] S. Solar, N. Getoff, P.S. Surdhar, D.A. Armstrong, A. Singh, Oxidation of tryptophan and N-methylindole by N₃[•], Br₂^{•-}, and (SCN)₂^{•-} radicals in light- and heavy-water solutions: a pulse radiolysis study, *J. Phys. Chem.*, 95 (1991) 3639-3643.
- [25] L. Nag, A. Lukacs, M.H. Vos, Short-Lived Radical Intermediates in the Photochemistry of Glucose Oxidase, *ChemPhysChem*, 20 (2019) 1793-1798.
- [26] S. Sharma, K. Sivalingam, F. Neese, G.K.-L. Chan, Low-energy spectrum of iron–sulfur clusters directly from many-particle quantum mechanics, *Nat. Chem.*, 6 (2014) 927-933.
- [27] H.A. Dailey, M.G. Finnegan, M.K. Johnson, Human ferrochelatase is an iron-sulfur protein, *Biochemistry*, 33 (1994) 403-407.
- [28] G. Sainz, J. Jakoncic, L.C. Sieker, V. Stojanoff, N. Sanishvili, M. Asso, P. Bertrand, J. Armengaud, Y. Jouanneau, Structure of a [2Fe–2S] ferredoxin from *Rhodobacter capsulatus* likely involved in Fe–S cluster biogenesis and conformational changes observed upon reduction, *J. Biol. Inorg. Chem.*, 11 (2006) 235-246.
- [29] D.W. Cushman, R.L. Tsai, I.C. Gunsalus, The ferroprotein component of a methylene hydroxylase, *Biochem. Biophys. Res. Comm.*, 26 (1967) 577-583.

- [30] J.J. Warren, T.A. Tronic, J.M. Mayer, The Thermochemistry of Proton-Coupled Electron Transfer Reagents and its Implications, *Chem. Rev.*, 110 (2010) 6961-7001.
- [31] M. Schütz, M. Brugna, E. Lebrun, F. Baymann, R. Huber, K.-O. Stetter, G. Hauska, R. Toci, D. Lemesle-Meunier, P. Tron, C. Schmidt, W. Nitschke, Early Evolution of Cytochrome *bc* Complexes, *J. Mol. Biol.*, 300 (2000) 663-675.
- [32] E.J. Leggate, J. Hirst, Roles of the Disulfide Bond and Adjacent Residues in Determining the Reduction Potentials and Stabilities of Respiratory-Type Rieske Clusters, *Biochemistry*, 44 (2005) 7048-7058.
- [33] T. Merbitz-Zahradnik, K. Zwicker, J.H. Nett, T.A. Link, B.L. Trumpower, Elimination of the Disulfide Bridge in the Rieske Iron–Sulfur Protein Allows Assembly of the [2Fe-2S] Cluster into the Rieske Protein but Damages the Ubiquinol Oxidation Site in the Cytochrome *bc*₁ Complex, *Biochemistry*, 42 (2003) 13637-13645.
- [34] M.Z. Hoffman, E. Hayon, One-electron reduction of the disulfide linkage in aqueous solution. Formation, protonation, and decay kinetics of the RSSR⁻ radical, *J. Am. Chem. Soc.*, 94 (1972) 7950-7957.