Investigation of the pH-dependence of the oxidation of FAD in VcCRY-1, a member of the cryptochrome-DASH family

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Abstract

Vibrio cholerae cryptochrome-1 (VcCRY-1) is a member of the cryptochrome DASH family. The flavoprotein appears to use blue light both for repair of cyclobutane pyrimidine dimers (CPDs) on DNA and signal transduction. Earlier, we found that it was almost impossible to oxidize the FADH[•] state upon binding to a CPD, and, in the absence of substrate, the rate of FADH[•] oxidation was much larger at high pH (Gindt et al. in *Biochemistry* 54:2802–2805, 2015). Here, we present the pH-dependence of the oxidation of FADH[•] by ferricyanide, which revealed a switch between slow and fast oxidation with a $pK_a \approx 7.0$. Stopped-flow mixing was used to measure the oxidation of FADH[•] to FADH⁺ at pH 6.7 and 7.5. Substrate binding was required to slow down this oxidation such that it could be measured with stopped flow, but there was only a small effect of pH. In addition, resonance Raman measurements of FADH[•] in VcCRY-1 at pH 6.5 and 7.5 were performed to probe for structural changes near the FAD cofactor related to the observed changes in rate of FADH[•] oxidation. Only substrate binding seemed to induce a change near the FAD cofactor that may relate to the change in oxidation kinetics. The pH-effect on the FADH[•] oxidation rate, which is rate-limited by the proton acceptor, does not seem to be due to a protein structural change near the FAD cofactor. Instead, a conserved glutamate in CRY-DASH may control the deprotonation of FADH[•] and give rise to the pH-effect.

Keywords Cryptochrome-DASH · DNA repair · FAD oxidation · Kinetics · Absorption spectroscopy · Raman spectroscopy

1 Introduction

The cryptochrome-DASH (CRY-DASH) subfamily is a member of the cryptochrome/photolyase family (CPF) of flavoproteins [1–5]. In addition to CRY-DASH proteins, the CPF is further comprised of cyclobutane pyrimidine dimer (CPD) and (6–4)–photolyases (PL) that repair CPDs and (6–4)–pyrimidine pyrimidone photoproducts on DNA, respectively, along with the plant and animal cryptochromes (CRY) that are involved in signaling in plant growth and regulation of the circadian clock [2–7]. All members of the CPF bind the required active site flavin adenine dinucleotide

(FAD) cofactor in their N-terminal domain, and, in addition, many members of the CPF family bind a second chromophore that seems to act as a light-gathering antenna [3]. DNA binding and repair occur at the N-terminal domain in PLs and CRY-DASH. Although CRYs have a photolyase homology domain in their N-terminal domain, they do not repair DNA. The CRYs do possess a C-terminal domain that varies in length and sequence between CRYs and is important for their signaling function. CRY-DASH has retained a non-canonical DNA-repair function but also has a signaling function, and is believed to be an evolutionary intermediate between PL and CRY [8].

All members of CPF use light absorption by the required FAD cofactor for their function, though CRYs in higher animals can be involved in entrainment of the circadian rhythm in a light-independent way [2–7]. In PL, the active state is the fully reduced FADH[•], and the enzyme cycles through the neutral radical FADH[•] state during its photocatalytic repair function [3]. PL in the FADH[•] state can be photoactivated (i.e. photoreduced) by light to form the active FADH[•] state, though the fully oxidized FAD (FAD_{ox}) state

Pushing the limits of flash photolysis to unravel the secrets of biological electron and proton transfer - a topical issue in honour of Klaus Brettel.

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is inactive. The CRY proteins appear to have a fully oxidized FAD_{ox} as their dark state while absorption of light creates the fully reduced or semi-reduced active states [9, 10]. Although the inactive FAD_{ox} in PL can be photoreduced to the anionic radical $\text{FAD}^{\cdot-}$, protonation to $\text{FADH}^{\cdot}$ is six orders in magnitude slower than in plant CRY [11]. Since CRY-DASH appears to have both DNA-repair and signaling functions [2, 8, 12–18], it presumably needs to control three FAD redox states compared to the other members of the CPF family that appear to control only two redox states. In addition, CRY-DASH may have to establish a functionally relevant photoreduction of FAD_{ox} , unlike the PL systems.

Although all redox states of the FAD cofactor are accessible under specific in vitro conditions, regulation of the redox chemistry of the FAD cofactor is key in establishing the specific function of each member of the CPF in vivo [19]. The protein environment of the FAD cofactor appears to regulate the cofactor by influencing the (de)protonation of the N(5) of the FAD by stabilizing the protonated N(5), by (de)stabilizing $\text{FADH}^{\cdot-}$ and by regulating the reduction potentials of the FAD cofactor. The amino acid proximal to the N(5) position of the FAD cofactor can vary with subfamily but is conserved within the subfamily. In the PL subfamily which cycles between $\text{FADH}^{\cdot-}$ and $\text{FADH}^{\cdot}$ during catalytic DNA-repair, it is an asparagine that stabilizes the N(5)–H through hydrogen bonding [20–22]. In the plant CRY subfamily with an FAD_{ox} dark state and $\text{FADH}^{\cdot}$ signaling state, it is an aspartate that may donate the proton to the FAD N(5) to form $\text{FADH}^{\cdot}$ from $\text{FAD}^{\cdot-}$ along with destabilizing a negatively charged $\text{FADH}^{\cdot-}$ [23–25]. The proximal amino acid in the animal CRY subfamily is a cysteine which appears to allow formation of an $\text{FAD}^{\cdot-}$ signaling state from the FAD_{ox} dark state, though this debate has not been settled [26–28]. The CRY-DASH subfamily has an asparagine, just like PL [29], which raises the question how CRY-DASH accesses the presumptive FAD_{ox} dark-state for its signaling function, if that is a requirement, and how it returns back to $\text{FADH}^{\cdot}$ and $\text{FADH}^{\cdot-}$ for its DNA-repair function.

While a single amino acid substitution may allow some promiscuity in the protein function, the role of the protein in regulation of the FAD cofactor is more complicated. So, while the aspartate to asparagine point mutation proximal to the N(5)–H in the FAD for the plant cryptochrome-1 from *Arabidopsis thaliana* (AtCRY-1) allowed the protein to display some PL-like DNA repair [23], the polarity of the amino acid one upstream from the N(5) proximal residue also seems to affect $\text{FADH}^{\cdot-}$ stability [30]. This residue is generally more polar in PL and plant CRY and mostly non-polar in animal CRY; it appears that a more polar residue stabilizes the $\text{FADH}^{\cdot-}$ state. In the CRY-DASH subfamily, it is a serine, as observed in most plant CRY. Holub, Kubař, Mast et al. have an extensive discussion on the importance of the FAD binding pocket in fine-tuning the function of the protein [19].

The electrochemistry of FAD is also affected by protein environment and substrate binding. For example, in

Escherichia coli CPD photolyase (EcPL) and in *Vibrio cholerae* cryptochrome-1 (VcCRY-1), a CRY-DASH, we found that the $\text{FADH}^{\cdot-}/\text{FADH}^{\cdot}$ reduction potential increased in the presence of substrate DNA in both proteins, while it is 164 mV higher in VcCRY-1 compared to EcPL at pH 7 [13, 31]. We have proposed that perturbation of hydrogen bonding induced by substrate binding between the FAD cofactor and the protein contributes to the increase of the $\text{FADH}^{\cdot-}/\text{FADH}^{\cdot}$ reduction potential and, in turn, to increased $\text{FADH}^{\cdot-}$ stability in an aerobic environment [31, 32]. We also determined that there was no pH-dependence of the $\text{FADH}^{\cdot-}/\text{FADH}^{\cdot}$ reduction potential in EcPL between pH 5.5–10 [33]. Brettel and coworkers found similar $\text{FADH}^{\cdot-}/\text{FADH}^{\cdot}$ reduction potentials in PL and a value significantly more negative in the plant CRY AtCRY-1 [34].

Brettel and coworkers showed that the yield of the signaling state $\text{FADH}^{\cdot}$ in the plant CRY AtCRY-1 has a pH-dependence with an estimated pK_a of 7.4; the $\text{FADH}^{\cdot}$ yield is higher at lower pH values [35]. In the presence of ATP, the $\text{FADH}^{\cdot}$ yield improves significantly and is pH-independent to at least pH 9. Our group has identified a potential pH- and substrate-dependence of the electrochemistry in CRY-DASH VcCRY-1 [36]. Chemical oxidation of $\text{FADH}^{\cdot}$ in VcCRY-1 occurred much faster at pH 7.75 than pH 6.30. In addition, $\text{FADH}^{\cdot}$ was stable against chemical oxidation for hours in the presence of ssDNA substrate with a CPD at both high and low pH. Under these conditions, a strong temperature dependence on this oxidation rate was observed. Based on these observations, we hypothesize that the function of CRY-DASH, i.e., DNA repair and signaling, may be controlled by a group that is sensitive to substrate binding and/or pH. Such control is facilitated by the fact that $\text{FADH}^{\cdot}$ oxidation requires electron and proton transfer of which the latter is the rate-limiting step. The protonation or deprotonation of a group—FAD or amino acid—may be strictly regulated by the protein to lock in a specific FAD redox state and, thereby, in a specific function.

In this work, we investigate both the $\text{FADH}^{\cdot}$ and $\text{FADH}^{\cdot-}$ oxidation in VcCRY-1 as a function of pH and in the absence and presence of substrate DNA. Unlike in most other studies of this type, we look at the recovery of the dark state and stability of the signaling state rather than the formation of the signaling state. Since $\text{FADH}^{\cdot}$ is presumably the common redox state in both functions of CRY-DASH, we measured the resonance Raman spectrum of the $\text{FADH}^{\cdot}$ in VcCRY-1 to test whether its interactions with the protein environment are affected by pH and/or substrate binding.

2 Experimental methods

2.1 Enzyme preparation

Overexpression and purification of VcCRY-1 was carried out as described earlier [13]. The cell line was the kind gift of Dr. Aziz Sancar. The concentrated purified protein was

immediately aliquoted into small tubes and stored in 0.4 M potassium sulfate and 20 mM potassium phosphate pH 7 at -80°C until use. The purified protein contains an additional maltose binding domain; this domain was not removed for these studies since earlier work showed no effect on DNA repair with its cleavage [13]. The FAD cofactor in our preparation of VcCRY-1 is present in three redox states; FADH^- (50–60%), $\text{FADH}^{\cdot}$ (5–10%) and FAD_{ox} (30–45%). The percent of each FAD redox state in various preparations was determined as described earlier [13].

2.2 Preparation of substrate

An oligonucleotide, p(dT)_{10} (Trilink Biotechnologies), was damaged in a 5 mM potassium phosphate solution at pH 7 with UV-light as described previously [32]. The prepared UV-damaged oligonucleotide, UV-p(dT)_{10} was stored at -20°C until use.

2.3 Kinetic experiments

The oxidation of the $\text{FADH}^{\cdot}$ state to FAD_{ox} by excess potassium ferricyanide was measured under aerobic conditions as a function of pH using the following procedure. Solutions containing the potassium ferricyanide and protein were incubated at 20°C prior to mixing. The reaction was initiated by hand mixing the solutions in a cuvette and monitoring the absorbance of the $\text{FADH}^{\cdot}$. Upon mixing, the solution contained 16.7 mM potassium ferricyanide, 88 mM K_2SO_4 , $\sim 40\text{ }\mu\text{M}$ VcCRY-1 in the FADH^- state, and 20 mM potassium phosphate buffer of the appropriate pH. The oxidation of FADH^- to $\text{FADH}^{\cdot}$ occurred too rapidly to observe under the conditions of this experiment. The absorbance data were collected on a Perkin-Elmer Lambda 5 thermostatted at 20°C with a monitoring wavelength of 595 nm with a 2 nm slit width and 1 s averaging time. The experiments at each pH were repeated two to three times. Kinetic traces at the same pH were fit to a series of two to three exponential terms using global analysis in Igor Pro (Wavemetrics) to get the observed rate constants. The residuals from the fit were used to determine the number of exponential terms required to describe the data. Since all reactions were completed with the identical potassium ferricyanide concentration, no correction was done for the pseudo-first order condition.

The oxidation of the $\text{FADH}^{\cdot}$ state to FADH^+ by excess potassium ferricyanide under aerobic conditions was measured at different pH values using stopped-flow mixing with absorption spectroscopy at 5°C . A solution containing potassium ferricyanide was mixed in a 1 to 1 ratio with the protein (with or without DNA substrate) solution using a SFA-20 stopped flow accessory (Hi-Tech Scientific) with a Varian Cary 300 spectrophotometer. The reaction was monitored at 590 nm with a 4 nm slit width and a 33 ms

averaging time. The syringes containing unreacted solution, the stopped flow mixer, and the cuvette holder were all thermostatted to 5°C . The reaction mixture contained varying concentrations of potassium ferricyanide, 88 mM K_2SO_4 , 20 mM phosphate buffer at a pH of either 6.7 or 7.5, and $\sim 25\text{ }\mu\text{M}$ FADH^- . In the absence of DNA substrate, UV-p(dT)_{10} , the reaction was too fast to observe with the stopped flow mixer. A $7\times$ molar excess of UV-p(dT)_{10} incubated with the protein in the dark prior to mixing was found to slow down the reaction sufficiently for measurement. The potassium ferricyanide concentration was in excess and varied from 1.0 mM to 0.33 mM. Approximately 10 traces were collected and averaged together for each potassium ferricyanide concentration. The averaged traces were fit to a double exponential using global analysis in Igor Pro (Wavemetrics) as determined from the fit residuals, taking into account the pseudo-first order condition of the ferricyanide concentration to get the second order rate constant of the reaction.

2.4 Resonance Raman experiments

All samples were measured in Raman spinning cells that were sealed with a septum. Prior to the experiment, VcCRY-1 was exchanged in the appropriate buffer using microconcentrators (Amicon Ultra 30 K); 0.4 M K_2SO_4 , 20 mM potassium phosphate at pH 6.5 or at pH 7.5. The VcCRY-1 concentration was between 200 and $220\text{ }\mu\text{M}$ in a $200\text{--}250\text{ }\mu\text{L}$ solution. The $\text{FADH}^{\cdot}$ form of VcCRY-1 was prepared as described previously [13]. For the experiments with substrate, UV-p(dT)_{10} was added in the dark between the dithionite reduction and the ferricyanide oxidation steps to a final concentration of about $600\text{ }\mu\text{M}$, a threefold excess. During the experiments, samples were cooled to $2\text{--}8^{\circ}\text{C}$ by a cold stream of dry air that was passed through an NaCl–ice bath.

The resonance Raman (rR) experiments were collected by excitation with 20 mW from a diode-pumped solid-state (DPSS) laser at 532 or 561 nm (UltraLasers). The laser light was focused in the sample with an $f=50\text{ mm}$ quartz lens. The Raman scattered light was collected at a 90° angle with an $f=50\text{ mm}$, $f/1.2$ camera lens (Nikkor, Nikon), and Rayleigh scattering was removed by using a 532 nm holographic notch filter (Kaiser Optical) or a 561 nm holographic notch filter (ThorLabs, Inc.). The Raman scattered light was focused ($f=200\text{ mm}$ quartz lens) through a wedge depolarizer (CVI Lasers Corp.) into a Triax 320 spectrometer (JY/Horiba) and was dispersed with a 2400 mm^{-1} groove grating and detected with a $\text{N}_2(\text{l})$ -cooled, front-illuminated, open-electrode CCD detector (Symphony, JY/Horiba). The rR spectra were calibrated with the known spectrum of toluene, and OriginPro 9 (OriginLab) was used for data analysis. Since $\text{FADH}^{\cdot}$ in VcCRY-1 was prepared by chemical titration, low and undetectable concentrations of FADH^- and/

or FAD_{ox} may have been present in the sample. A relatively high but structureless background due to luminescence from FAD_{ox} and $\text{FADH}^\cdot$ lowered the signal-to-noise ratio of the rR spectra and was corrected for by subtracting a polynomial that was fitted to the baseline. Spectra before and after correction were compared to avoid the generation of artifacts.

3 Results

VcCRY-1 is isolated with the FAD chromophore mainly in the fully reduced state but all three oxidation states are present [13]. Upon thawing from -80°C storage, a typical preparation has 50–60% of the protein in the FADH^- state, 5–10% in the $\text{FADH}^\cdot$ state with the remainder in the FAD_{ox} state. The protein could be stored on ice in pH 7.0 buffer for multiple hours without any further oxidation observed.

3.1 Investigation of the pH-dependence for the oxidation of the $\text{FADH}^\cdot$ state

In earlier work, we reported that at a pH of 6.3, the oxidation of $\text{FADH}^\cdot$ by excess potassium ferricyanide was dramatically slower than the same oxidation at a pH of 7.75 [36]. We reproduced this dramatic pH effect on the rate of the oxidation, as shown in Fig. 1. Of the three oxidation states found in VcCRY1, only the $\text{FADH}^\cdot$ state absorbs light at 595 nm while the ferricyanide oxidant used and its ferrocyanide product absorb below 450 nm. Therefore, any changes to the 595 nm absorbance reflect changes in the concentration of the $\text{FADH}^\cdot$ state of the protein.

To obtain the kinetic traces shown in Fig. 1, the oxidation of VcCRY-1 was induced by hand mixing the protein with $\sim 400\times$ molar excess of potassium ferricyanide, all at 20°C . Oxidation of FADH^- to $\text{FADH}^\cdot$ occurs too rapidly to detect in this particular experiment, so only the loss of the $\text{FADH}^\cdot$ state, presumptively to FAD_{ox} , is observed in the trace. Using excess potassium ferricyanide as the oxidant gave a large starting population of the $\text{FADH}^\cdot$ state and greatly improved the signal to noise of the experiment. Three representative traces (pH 6.75, 7.00, and 7.50) are shown to illustrate the pH-dependence of the oxidation kinetics. The trace collected at a pH of 6.75 required 19 min for a 50% decrease in the absorbance at 595 nm while the same decrease for the pH of 7.0 and 7.5 required 7.2 and 1.5 min, respectively. An even more dramatic effect on the rate of oxidation is shown by the sample that contains a bound UV-p(dT)₁₀ substrate where more than 30 min is required for a 50% decrease in the signal at pH of 7.5; this effect of substrate is observed throughout the pH range. We were unable to obtain a good kinetic trace with oxidation of $\text{FADH}^\cdot$ at low pH with substrate bound, because the oxidation proceeded too slowly, i.e., over the course of multiple days.

Fig. 1 The typical kinetic traces monitoring the oxidation of the $\text{FADH}^\cdot$ state in VcCRY-1. The oxidation was initiated by addition of potassium ferricyanide to VcCRY-1. The reaction is monitored at 595 nm at 20°C with an averaging time of 1 s per point. The reaction mixture started with 16.7 mM potassium ferricyanide, 88 mM K_2SO_4 , $\sim 40\ \mu\text{M}$ VcCRY-1 in the FADH^- state, and 20 mM phosphate buffer of the appropriate pH. A slight molar excess (1.2× molar equivalence) of UV-p(dT)₁₀ was added to the protein prior to mixing with oxidant, if applicable. Oxidation of the initial FADH^- state was too fast for the time resolution of the experiment so only the oxidation of the $\text{FADH}^\cdot$ state was observed

Oxidation of the $\text{FADH}^\cdot$ state was measured from a pH of 6.5 to 7.75 to determine the transition point of the change in the kinetics. Multiple trials were collected at each pH studied, and the traces were fit to a series of exponentials using global analysis. At pH less than 7, the traces required three exponentials to describe the data, as judged from the residuals: the observed rate constants were $\sim 0.04\ \text{min}^{-1}$ ($\sim 80\%$ of the signal), $\sim 0.15\ \text{min}^{-1}$ (less than 10% of the signal) and $\sim 10\ \text{min}^{-1}$ ($\sim 15\%$ of the signal). At pH above 7.1, only two exponentials were required: $\sim 0.4\ \text{min}^{-1}$ ($\sim 80\%$ of the signal) and $\sim 10\ \text{min}^{-1}$ ($\sim 20\%$). In Fig. 2, we focused on the rate constants that contributed more than 75% of the signal: $\sim 0.04\ \text{min}^{-1}$, the majority component below pH 7, and $\sim 0.4\ \text{min}^{-1}$, the majority component above pH 7. We believe that the $0.15\ \text{min}^{-1}$ component below pH 7 and the $0.40\ \text{min}^{-1}$ component above pH 7 reflect the same process with a small pH-sensitivity. The fast rate ($10\ \text{min}^{-1}$) that is observed in all kinetic traces is most likely a mixing artifact. A plot of the percent amplitude to the total change as a function of pH shows a sigmoidal changeover between the rate constants at neutral pH. The pH-dependence in the

Fig. 2 The pH-dependence on the oxidation kinetics of the FADH· state. Multiple traces were collected by monitoring the 595 nm absorbance at each pH and analyzed using global analysis; generally, a double exponential or triple exponential was needed to describe the data. The contribution of the major component to the trace by amplitude was calculated from the global analysis results and plotted as a function of pH. The observed rate constants have not been corrected for pseudo-order conditions with the ferricyanide concentration

graph is very similar to behavior expected with a protonation/ deprotonation of an amino acid, though the transition seems quite narrow for a single contributor. It should be noted that the initial absorbance of FADH· of the kinetic traces was, within experimental error, consistent with the sum of the original amount of FADH· present in the enzyme plus the amount FADH· that was formed from rapid oxidation of FADH⁻ to FADH·, a step that was not resolved in the hand-mixing experiments. This observation shows that we did not miss any fast FADH· oxidation step in the hand-mixing experiment.

When the experiments were performed under aerobic conditions at 20 °C without potassium ferricyanide, nearly identical rates of FADH· oxidation, within experimental error, and the same pH-dependence were observed with oxygen as the only oxidant present. The rate of FADH· oxidation to FAD_{ox} appears to be independent of oxidant used. This strongly suggests that the FADH· oxidation kinetics are rate-limited by the protonation step. In the absence of potassium ferricyanide and under nearly anaerobic conditions, only limited FADH· oxidation was observed. In addition, we rule out H₂PO₄⁴⁻ or HPO₄²⁻ as the putative proton donor as changing the H₂PO₄⁴⁻/HPO₄²⁻ concentration by a factor of 10 did not alter the FADH· oxidation kinetics at both high and low pH (data not shown).

The rate of the oxidation reaction was measured with a range of concentrations (from 8 to 40 μM) of protein at an acidic, neutral, and basic pH. The protein concentration did not have an effect on the observed rate constants; we

do not see any indication of oligomeric structure formation as a function of pH.

3.2 Investigation of the effect of pH on oxidation of the FADH· state

To gain more insight into the possible origins of this dramatic change in the kinetics of the oxidation of the FADH· state, experiments were designed to examine the oxidation of the FADH⁻ to FADH· at low and high pH. Since the oxidation of the fully reduced state is much faster than that of the semiquinone state, stopped flow mixing was used to initiate the reaction, and the absorbance of the product of the oxidation, the FADH· state, was monitored. Large quantities of protein were required for these experiments, so the reaction was monitored at only two pH values: 6.7 and 7.5. The reaction was run under pseudo-first order conditions with potassium ferricyanide in large excess. The reaction between the VcCRY-1 FADH⁻ state and ferricyanide occurred within the mixing time of the stopped-flow instrument at 5 °C even after the concentration of ferricyanide was decreased to the lowest concentration limit of pseudo-first order kinetics. Given the slowing effect observed with substrate binding on the oxidation kinetics of the FADH· state, experiments were designed to add UV-p(dT)₁₀ substrate in a 7 times molar excess to the protein prior to mixing; this solution was kept in the dark to prevent repair of the substrate. The presence of the DNA substrate slowed down the oxidation kinetics sufficiently, as shown in Fig. 3, to enable measurement of the oxidation of the FADH⁻ state.

Three different concentrations of the ferricyanide oxidant were used at each pH with approximately 10 traces captured for each set of experimental conditions. The individual traces were averaged, and the three concentration dependent traces for each pH were fit using global analysis with pseudo-first order kinetics to extract the best second-order rate constant from the entire data set. The second order rate constants extracted from global analysis are 16.6 (±0.1) and 6.47 (±0.03) s⁻¹ mM⁻¹ for pH 6.7 and 14.0 (±0.1) and 4.40 (±0.02) s⁻¹ mM⁻¹ for pH 7.5. Unlike what is observed with oxidation of the FADH· state, the fully reduced state does not show a large pH-dependence. This observation would be consistent with no large pH-induced changes in the protein structure around the cofactor. The oxidation of FADH⁻ to FADH· requires only electron transfer, not proton transfer. Therefore, inaccessibility of a proton donor/ acceptor would not be expected to show up in this step. Each data set required a double exponential, as judged from the residuals of the fit.

Fig. 3 The typical kinetic traces monitoring the oxidation of the FADH^- state in VcCRY-1 in the presence of DNA substrate. The oxidation was initiated by addition of potassium ferricyanide to VcCRY-1 using stopped flow mixing. The reaction was monitored at 595 nm at 5 °C with an averaging time of 33 ms per point. The reaction mixture started with 1.0 mM potassium ferricyanide, 88 mM K_2SO_4 , ~40 μM VcCRY-1 in the FADH^- state, and 20 mM phosphate buffer of the appropriate pH. A 7× molar excess of UV-p(dT)₁₀ was added to the protein prior to mixing with oxidant; the protein solution was kept dark prior to the reaction to prevent repair of substrate. The oxidation was too rapid in the absence of DNA substrate to measure using stopped flow mixing

3.3 Resonance Raman experiments on the effect of pH and substrate binding on FADH^-

The resonance Raman spectra of FADH^- in VcCRY-1 at pH 6.5 and 7.5 in the absence and presence of UV-p(dT)₁₀ with excitation at 532 and 561 nm are shown in Fig. 4A, B, respectively. The spectra are similar to those that we reported earlier for FADH^- in VcCRY-1 at pH 7 [13] with Raman band positions differing by 1 cm^{-1} or less. The resolution of the current spectra is better because no x -axis binning of the CCD detector was used during data collection. The improved resolution provides clear and reproducible differences between the rR spectra of FADH^- in VcCRY-1 and FADH^- in VcCRY-1 with UV-p(dT)₁₀ at both pH values and at both excitation wavelengths, though the differences are clearer with 561 nm excitation. Figure 5 shows an enlargement of the rR spectra obtained with 561 nm excitation with significant differences observed in three regions of the Raman spectrum; 1220–1250 cm^{-1} , 1300–1350 cm^{-1} and 1370–1410 cm^{-1} .

In the 1220–1250 cm^{-1} region, the addition of UV-p(dT)₁₀ to VcCRY-1 causes no change in the vibrational mode at 1227 cm^{-1} , but the vibrational mode at 1238 cm^{-1} shifts to 1241 and 1242 cm^{-1} at pH 6.5 and 7.5, respectively. Although the peak intensity is low, the change from being a shoulder at 1238 cm^{-1} on the 1227 cm^{-1} peak to a resolved peak at 1241/1242 cm^{-1} is clear. In the 1300–1350 cm^{-1} region, the band at 1303 cm^{-1} shifts to 1305 cm^{-1} when substrate is present. In the presence of UV-p(dT)₁₀, a small band becomes visible at 1344 cm^{-1} that is weaker and/or more overlapping with the 1331 cm^{-1} band in the absence of substrate. The clearest difference is observed in the 1370–1410 cm^{-1} region. The addition of UV-p(dT)₁₀ causes a shift in the 1390 cm^{-1} peak to 1393 cm^{-1} . The adjacent 1378 cm^{-1} peak is not affected but becomes better resolved in the presence of UV-p(dT)₁₀. Control experiments on UV-p(dT)₁₀ in buffer and on fully reduced and fully oxidized VcCRY-1 did not show any Raman bands that were sufficiently strong to affect the rR spectra of FADH^- and do not account for the observed differences between substrate-free and substrate-bound VcCRY-1 FADH^- (data not shown). In addition, these differences are similar to those that we observed before upon substrate binding in EcPL and VcCRY-1 [13, 32, 37], and they have been confirmed in EcPL by others [38].

4 Discussion

CRY-DASH proteins seem to isolate as a mixture of all three oxidation states with the fully reduced state as the most prevalent [13, 39]. This is in contrast to PLs which are usually isolated as FADH^- or as a mixture of $\text{FADH}^-/\text{FADH}^-$ and the CRY proteins which are isolated in the FAD_{ox} state. Since most of VcCRY-1 is already present as FADH^- , formation of the more oxidized states is highly relevant to its presumptive role in signaling where the dark state seems to be FAD_{ox} . Although potassium ferricyanide and oxygen give the same FADH^- oxidation kinetics, we used potassium ferricyanide rather than the more biologically relevant dissolved O_2 gas to control the oxidation reactions more fully. In addition, the rapid oxidation of FADH^- to FADH^- by potassium ferricyanide within the mixing time of the experiment resulted in a significantly larger initial FADH^- absorbance that facilitated the experiments and gave a better signal-to-noise ratio.

As reported earlier, we see a dramatic effect of the pH on the rate of the oxidation of the FADH^- to form FAD_{ox} [36], which requires the removal of both an electron and a proton from the cofactor. Damiani, Yalloway, Lu et al. presented evidence that this step requires proton-coupled electron transfer [39]. Therefore, the dramatic pH effect on this oxidation rate is most likely due to the availability of a proton acceptor to the N(5)-H at pH > 7. It may be possible that

Fig. 4 Resonance Raman spectra of VcCRY-1 FADH $\cdot$ obtained with 532 nm **A** and 561 nm **B** excitation. Spectra were collected at pH 6.5 in the absence (a) and presence (b) of UV-p(dT) $_{10}$ and at pH 6.5 in the absence (c) and presence (d) of UV-p(dT) $_{10}$

the proton donor for the photoreduction of the FAD group will also work as the proton acceptor for the oxidation of the FADH $\cdot$. Since our results show that FADH $\cdot$ oxidation is independent of oxidant, the deprotonation of the FAD N(5)H seems to be the rate-limiting step. In the photoreduction of plant CRY FAD $_{\text{ox}}$, the group donating a proton to the FAD is an aspartate; the homologous residue in the CRY-DASH family is an asparagine, a group unable to donate a proton [29]. Figure 6 displays some of the amino acids around the FAD in the CRY-DASH AtCRY-3 [40]. Recent computational work proposes that the proton donor for the same photoreduction in PL is likely a water molecule, and that water molecule is part of a “water wire”, a series of water molecules that connect the FAD binding pocket to the bulk solvent [19]. However, photoreduction of FAD $_{\text{ox}}$ to FADH $\cdot$ in EcPL has a protonation step that is six orders in magnitude slower than in plant CRY [11].

In our work, we see two experimental conditions that significantly affect the oxidation of the FADH $\cdot$ in VcCRY-1: pH and presence of damaged DNA substrate. Applying the hypothesis of a “water wire” could explain the decrease in the oxidation rate with DNA substrate bound; presence of the substrate could disrupt the “water wire” to the bulk solvent as discussed in more detail below. The pH effect is more difficult to explain unless the protonation state of an amino acid along the proposed “water wire” ends up disrupting or stabilizing the formation of the water network. Holub, Kubař, Mast et al. identify two amino acids in EcPL

close to the proposed water wire: Met367 and Leu376 [19]. In the CRY-DASH sequences examined (Cryptochrome-3 from *A. thaliana* (AtCRY-3), *Synechocystis* sp. PCC6803, and VcCRY-1), the nonpolar methionine in EcPL is replaced by a charged glutamate ($\text{pK}_{\text{a}} \sim 4.3$), which may be conserved within the CRY-DASH subfamily, while the leucine is replaced by another nonpolar residue in the CRY-DASH proteins. In Fig. 6, using the crystal structure of AtCRY-3, Glu417 (the conserved glutamate) is shown relative to the FAD cofactor. It is plausible that the protonated glutamate might inhibit the “water wire” that is needed for the proton transfer away from the FADH $\cdot$ during the oxidation step; to be consistent with our data, this glutamate would have an elevated pK_{a} around 7 in the protein.

If the glutamate in the proposed ‘water-wire’ in CRY-DASH is the titratable group that causes the pH-dependence in the FADH $\cdot$ oxidation kinetics, it is likely too far away from the FAD isoalloxazine ring to induce a change, if any, in its protein environment. This is consistent with the fact that we did not observe any differences between the rR spectra of FADH $\cdot$ in VcCRY-1 at pH 6.5 and 7.5. Other explanations of the pH-dependence on the oxidation of the FADH $\cdot$ are plausible. For example, there could be a pH-dependent structural change that moves a proton acceptor closer to the N(5)H at higher pH. Therefore, we also probed the pH-dependence of the one electron oxidation of FADH $\cdot$ to form FADH $^-$, and we found only a minor effect, arguing against large structural changes around the cofactor

Fig. 5 Enlargement of the observed changes in the resonance Raman spectra of VcCRY-1 FADH• in the absence (black line) and presence (red line) of UV-p(dT)₁₀. The spectra were collected with 561 nm excitation at pH 6.5 (top) and pH 7.5 (bottom). All spectra were normalized on the 1606 cm⁻¹ peak that did not change with pH or upon substrate binding

Fig. 6 Relevant amino acids in the FAD binding pocket of a CRY-DASH protein. This figure was created with Pymol and used the crystal structure of AtCRY-3 (code 2J4D) with the numbering of this species [40]. The amino acids that are denoted are conserved throughout three CRY-DASH sequences examined (AtCRY-3, VcCRY-1, SynCRY-1)

as supported by the rR data. Since this oxidation step does not require a proton transfer, it is insensitive to changes to the protonation and/or H-bonding state of a glutamate in the proposed ‘water wire’.

In the presence of substrate, FADH• oxidation slows down considerably and still shows a large pH effect; above pH 7 oxidation takes hours and below pH 7 it takes days. Substrate binding also induces changes in the rR spectra of FADH• in VcCRY-1 that are independent of pH. The bands at 1238, 1303, 1344 and 1390 cm⁻¹ in VcCRY-1 that shift upon substrate binding are the same vibrational modes that we observed shifting in EcPL and VcCRY-1 at pH 7.0 [13, 32] and also have been observed by others [38]. We concluded that the shifts in the Raman bands were most likely due to changes in the H-bonding by N(5)H and N(3)H with the protein environment. With the crystal structure of *Anacystis nidulans* PL (AnPL) and AtCRY-3 resolved in the presence of substrate analogs, it is clear that the CPD has the potential to form hydrogen bonds with the adenine of the FAD cofactor [12, 41]. In a recent FTIR study on EcPL with substrate bound it was proposed that the CPD forms hydrogen bonds with the FAD adenine and with Glu275 in the CPD binding pocket, which corresponds to Glu325 in AtCRY-3 (Fig. 6) [42]. The Glu275 seems protonated when DNA is present and deprotonated when DNA is absent. In EcPL without substrate, Glu275 shares a water with an FAD phosphate group [43]. It seems that substrate-binding causes a direct interaction with the FAD cofactor at the adenine group and that Glu275 rotates to form a hydrogen bond with the CPD while losing its H-bond to the water through which it is H-bonded to the FAD phosphate. We propose that these changes in the H-bonding network surrounding the FAD trigger changes in H-bonding at the N(5)H and N(3)H that we detect with the rR spectroscopy. Previously, we proposed that these changes resulted in an increase in FADH⁻/FADH• reduction potential and, consequently, to a ~2-fold decrease in an electron transfer rate and ~10-fold stabilization of FADH⁻ [28, 44]. Such changes are insufficient to fully explain the order in magnitude change in the rate of FADH• oxidation in VcCRY-1 upon substrate-binding.

The most straightforward explanation for the substrate-binding effect on the rate constants of FADH⁻ and FADH• oxidation is that the substrate blocks access of the oxidizing agents—ferricyanide or oxygen—to the FAD cofactor. A similar effect has been observed before for FADH⁻ in EcPL with O₂ as the oxidant [45]. Despite its much smaller size, oxygen generally still requires one or more channels to access a buried active site within a protein as demonstrated by computer simulations of the pathway of O₂ in Mb [46], and cannot diffuse randomly through the protein. In prior work, we have analyzed the effect of substrate binding on electron transfer in EcPL [31, 44], and, for the large increase in the rate of FADH• oxidation in the presence of substrate,

the simplest explanation is an increase in electron transfer distance. However, the picture of FADH $\cdot$ oxidation is more complicated. Although the electron transfer step may be decreased by substrate binding, the presence of a large pH effect in FADH $\cdot$ oxidation indicates that the proton transfer step is also rate-limiting with substrate bound. The rR experiments provide evidence that substrate binding causes perturbation of the H-bonding network near N(5)H and N(3)H that may interfere with the deprotonation of the FAD N(5)H. Since the pH effect still occurs between pH 6.5 and 7.5, there is no dramatic change in the pK_a of the proton acceptor. It appears that substrate binding shuts down the deprotonation of the N(5)H, especially at and likely below pH 7.5. However, our current results do not provide sufficient information to identify any specific cause for the large substrate effect on FADH $\cdot$.

5 Conclusion

The CRY-DASH subfamily appears to be able to use light both for repair of UV-damaged ssDNA and for signal transduction. DNA repair requires the fully reduced FADH $^-$ active state along with a relatively stable FADH $\cdot$ state intermediate. Assuming a similar signaling mechanism to the plant and animal CRY proteins, the CRY-DASH protein would also need to have a stable FAD $_{ox}$ dark state with more reduced signaling states. The conversion between the two roles may hinge about the availability of a proton acceptor / donor to the N(5)H of FAD.

In this work, we find a switch in the rate of the oxidation of FADH $\cdot$ in VcCRY-1 around pH 7; the major contributing rate constant being 10 times slower at lower pH. It appears that the oxidation process is rate-limited by the proton transfer step, and the pH-dependence bears a strong resemblance to the (de)protonation of a group centered at a pH of 7.0. The absence of a pH-effect in the rR spectra of FADH $\cdot$ argues against the FAD cofactor or an amino acid interacting with it as the titratable group. Our analysis shows that the best candidate is an apparently conserved glutamate in CRY-DASH near a water wire similar to the one that was recently proposed in EcPL that may support very slow protonation of the FAD N(5) [19]. We propose that a similar water wire exists in CRY-DASH that supports the deprotonation of FADH $\cdot$. The glutamate, which would have an elevated pK_a of 7, could be the switch that locks the enzyme into its DNA-repair function below pH 7 and into its signaling function above that pH.

Substrate binding slows down the oxidation of both FADH $^-$ and FADH $\cdot$. The simplest explanation is that substrate-binding restricts the accessibility of oxidizing agents to the FAD-binding pocket and significantly slows down the electron transfer step in both oxidation processes.

However, the oxidation of FADH $\cdot$ in the presence of substrate is still rate-limited by the proton transfer step. The rR data show that substrate binding does cause perturbation of the H-bonding network involving the FADH $\cdot$ N(5)H and N(3)H regardless of pH. Although such perturbations can affect the FADH $^-$ /FADH $\cdot$ reduction potential and electron transfer rates [31, 43], these changes are too small to solely explain the large effect of substrate binding on the oxidation rates of FADH $^-$ and FADH $\cdot$ in VcCRY-1. The picture that emerges is that substrate binding locks VcCRY-1 in its DNA repair function by slowing down FADH $^-$ oxidation and all but completely inhibits FADH $\cdot$ oxidation, especially at a pH below 7. In the absence of substrate, a conserved glutamate may act as a switch to allow FADH $\cdot$ oxidation above pH 7 by providing a pathway for deprotonation of FADH $\cdot$, thereby reaching the presumed signaling dark state (FAD).

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Author contributions YMG and JPMS designed experiments, YMG overexpressed, isolated and purified protein, ALVH and YMG performed stopped-flow experiments, GC, YMG and MK performed hand-mixing experiments, JPMS performed resonance Raman experiments, YMG and JPMS interpreted data and wrote manuscript.

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Declarations

Conflict of interest The authors declare no conflicts or competing interests.

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