

Binding of Substrate Locks the Electrochemistry of CRY-DASH into DNA Repair

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Supporting Information

ABSTRACT: VcCry1, a member of the CRY-DASH family, may serve two diverse roles *in vivo*, including blue-light signaling and repair of UV-damaged DNA. We have discovered that the electrochemistry of the flavin adenine dinucleotide cofactor of VcCry1 is locked to cycle only between the hydroquinone and neutral semiquinone states when UV-damaged DNA is present. Other potential substrates, including undamaged DNA and ATP, have no discernible effect on the electrochemistry, and the kinetics of the reduction is unaffected by damaged DNA. Binding of the damaged DNA substrate determines the role of the protein and prevents the presumed photochemistry required for blue-light signaling.

The cryptochrome and photolyase families consist of structurally related flavin adenine dinucleotide (FAD) proteins that use the absorption of blue light to accomplish different tasks.¹ DNA photolyase (PL) uses the blue light for light-driven electron transfer to repair UV-induced cyclobutylpyrimidine dimers (CPD) on DNA molecules using the fully reduced hydroquinone state, FADH[−].² The cryptochromes (CRY) are blue-light photoreceptors involved in the circadian clock for plants and animals, and they use the fully oxidized FAD state.^{3–5} The CRY-DASH family of proteins appears to share sequence homology with both PL and CRY, with published reports that the CRY-DASH protein may serve a dual role; it may repair DNA under conditions of high light and serve as a photoreceptor under conditions of low light.^{6–9} In the course of our research, we have discovered that binding of UV-damaged DNA to CRY-DASH prevents the full oxidation of the cofactor to the FAD state, presumably restricting the role of the protein to exclusively DNA repair when CPD lesions are present.

Cryptochrome 1 from *Vibrio cholerae* (VcCRY1) has been identified as a member of the CRY-DASH family.^{10,11} Given its ability to bind and repair UV-damaged single-strand DNA,¹⁰ we initiated studies of the protein to understand the thermodynamics of the substrate binding and redox chemistry of the FAD cofactor in comparison to those of the better understood PL system.¹² There are three stable forms of FAD found with VcCRY1: the hydroquinone required for DNA repair, FADH[−], the neutral semiquinone that is part of the DNA repair cycle, FADH[•], and the fully oxidized form, FAD (the isoalloxazine moiety of FAD is shown in Figure 1A), with an unknown role in VcCRY1.

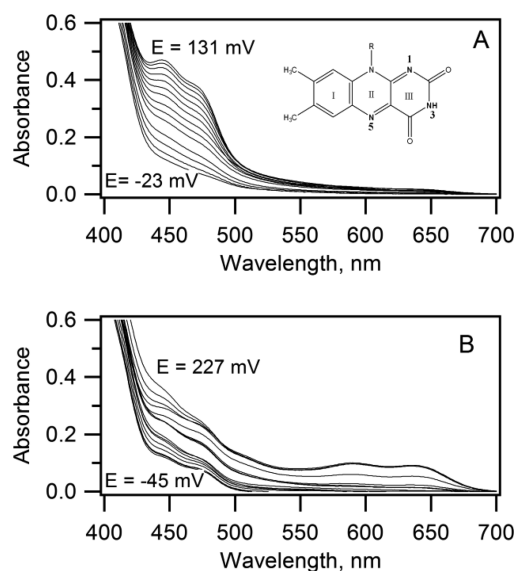


Figure 1. Spectroelectrochemical titrations of VcCRY1. The reaction mixture was prepared as described in the Supporting Information. The pH is 7.90, and $T = 10\text{ }^{\circ}\text{C}$. FAD appears at 443 and 470 nm; FADH[•] appears at 510, 595, and 640 nm. Fe(CN)₆^{3−}, the oxidant, and mediators absorb at 420 and ~450 nm, respectively. The highest and lowest solution potentials are indicated: (A) protein alone and (B) protein with UV-p(dT)₁₀. The structure of oxidized isoalloxazine is shown in panel A.

As discussed below, the redox chemistry of the VcCRY1 FAD is significantly different from that of the cofactor in PL, and these differences strongly indicate a mechanism by which the CRY-DASH family may be able to successfully navigate roles in both DNA repair and blue-light signaling *in vivo*.

Spectroelectrochemical titrations were initiated to measure the standard reduction potential, E° , of the FAD/FADH[•] couple using the method outlined by Dutton.¹³

The modification of the electrochemistry due to bound UV-damaged substrate is dramatically demonstrated at a high pH in Figure 1. In the experiment, the reduction potential of the solution was incrementally increased from −23 to 131 mV. Less than 10% of the VcCRY1 is detected in the FADH[•] state. The only difference between the spectra shown in panels A and B of Figure 1 is the presence of a 5-fold molar excess of UV-p(dT)₁₀; all other experimental conditions are identical. As the

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potential of the solution is changed from -45 to 227 mV in Figure 1B, the FADH^- state is transformed to stable FADH^\bullet and no further reaction occurs even at high potential. Oxidation of the FADH^\bullet cofactor to FAD is clearly blocked when the UV- $\text{p}(\text{dT})_{10}$ is present in the reaction mixture. There is no discernible effect of the UV- $\text{p}(\text{dT})_{10}$ substrate on the reduction of FAD to FADH^- at either pH, and the reduction occurs readily upon addition of dithionite.

The stability of the FADH^\bullet state is greater at pH 6.40, and the system is well behaved with clear single-electron transfer steps from FADH^- to FADH^\bullet to FAD. The same suppression of the oxidation to FAD is observed when a 5-fold molar excess of UV-damaged DNA is present. Incubation of the enzyme substrate complex in excessive concentrations of oxidant (solution potential of >200 mV) along with a reaction time of >20 h is not sufficient to produce FAD at 10°C .

In the absence of UV- $\text{p}(\text{dT})_{10}$, the E° for the $\text{FADH}^\bullet/\text{FAD}$ couple was found to be 209 ± 20 mV versus the normal hydrogen electrode at 10°C and pH 6.40 (data not shown). The standard reduction potential of VcCRY1 with the addition of a 5-fold molar excess of undamaged $\text{p}(\text{dT})_{10}$ or a 25-fold molar excess of ATP to the protein was indistinguishable from that obtained for the protein alone. In contrast, an earlier paper reported that the E° of the $\text{FADH}^-/\text{FADH}^\bullet$ couple of PL from *Escherichia coli* (EcPL) was virtually unaffected by pH in a range of 5.5–10 and the EcPL FADH^\bullet state was stable over the entire pH range.¹⁴

To better understand the kinetics of the oxidation from FADH^\bullet to FAD as a function of pH, substrate, and temperature, a series of experiments were completed using UV-vis absorption spectroscopy to monitor the FADH^\bullet state via the 595 nm absorbance. Each reaction mixture contained a 500-fold molar excess of potassium ferricyanide that should result in a potential of the solution of >450 mV along with pseudo-order kinetics for the oxidant. The reaction mixture turned green during mixing; the FADH^- state rapidly oxidized to FADH^\bullet prior to data acquisition. As shown in the Supporting Information, the reaction order for the ferricyanide was found to be $[\text{ferricyanide}]^{-1/3}$, a surprising result that may be linked to activity effects because higher concentrations of K_2SO_4 also slowed the oxidation reaction.

Figure 2 clearly demonstrates that oxidation of the FADH^\bullet to FAD can be dramatically slowed by two independent factors: (a) lowering the pH and (b) addition of UV-damaged DNA. Addition of ATP, GTP, or undamaged $\text{p}(\text{dT})_{10}$ had minimal impact on the oxidation kinetics at 20°C . We have evidence

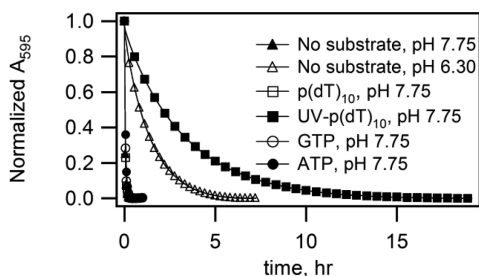


Figure 2. Oxidation of FADH^\bullet to FAD as monitored by loss of FADH^\bullet . Reactions were initiated by mixing of VcCRY1 with oxidant and buffer. All reaction mixtures initially contained 20 mM $\text{Fe}(\text{CN})_6^{3-}$, 88 mM K_2SO_4 , 20 mM phosphate, and 40 mM VcCRY1 (25 mM FADH^- , ~ 1 mM FADH^\bullet , or 16 mM FAD). $[\text{GTP}] = [\text{ATP}] = 500$ mM. $[\text{UV-p}(\text{dT})_{10}] = 170$ mM. $[\text{p}(\text{dT})_{10}] = 70$ mM. $T = 20^\circ\text{C}$.

that ATP binds to the more reduced states of VcCRY1 in stoichiometric amounts [isothermal titration calorimetry (ITC) data not shown]. We did not complete low-temperature or low-pH experiments with UV-damaged DNA because of the extreme times required. Examples of the large temperature dependence of the oxidation reaction are displayed in Figure 3,

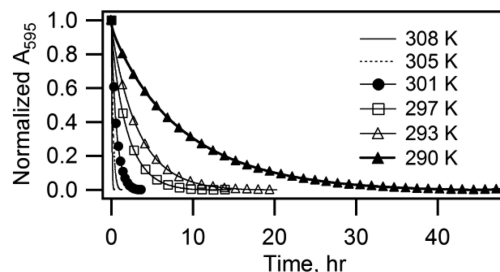


Figure 3. Temperature dependence of oxidation of FADH^\bullet to FAD with UV- $\text{p}(\text{dT})_{10}$ present. Conditions as in Figure 2.

with the kinetic traces of the oxidation in the presence of UV- $\text{p}(\text{dT})_{10}$. The traces were fit to either one or two exponentials; the χ^2 value and the residual of the fit were used to determine if two terms were required. The individual rate constants are reported in the Supporting Information. Only one exponential term was required for the oxidation at low pH. Two terms were consistently required for oxidation at high pH except for the highest temperature (35°C); the dominating term is always $>70\%$ of the total amplitude.

The Arrhenius plots for the FADH^\bullet oxidation at pH 7.75 and 6.30 along with the data for pH 7.75 with UV- $\text{p}(\text{dT})_{10}$ present are shown in Figure 4. For the high-pH work, only the rate

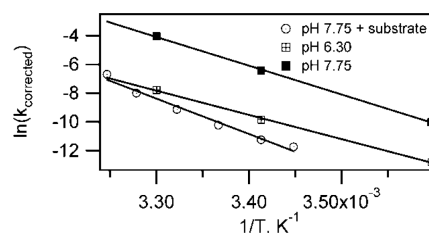


Figure 4. Arrhenius plots of the oxidation of FAD from FADH^\bullet . The rate constants were corrected for the dependence on ferricyanide concentration. Linear fits from least-squares analysis.

constant with the larger amplitude was used in the Arrhenius plot. The reaction at high pH had both a larger pre-Arrhenius factor ($e^{62} \text{ s}^{-1} \text{ M}^{-1}$) and activation energy (167 kJ/mol) compared to those of the reaction at low pH ($e^{48} \text{ s}^{-1} \text{ M}^{-1}$ and 140 kJ/mol, respectively), but both plots appear to be linear. The Arrhenius plot for the protein with substrate bound is more complicated with clear nonlinear behavior; the linear fit produced $A = e^{73} \text{ s}^{-1} \text{ M}^{-1}$ and $E_A = 205$ kJ/mol.

To test the difference between CRY-DASH and PL, the experiments at pH 7.75 were repeated with EcPL at 20°C ; the FADH^\bullet absorbance decreased in the first 3 min, showing oxidation of the stable semiquinone state, but then the magnitude of the signal dramatically increased (see the Supporting Information). The EcPL solution turned cloudy and green; the increase in absorbance at 595 nm after 3 min was due to light scatter. There was no discernible stabilizing effect on EcPL oxidation with UV- $\text{p}(\text{dT})_{10}$ present.

Overall, the VcCRY1 protein is much more stable in the oxidized state than EcPL. Some turbidity does develop with the VcCRY1 samples at high pH at 32 and 35 °C over time; the precipitation occurred after the FADH[•] had been completely lost, so we believe it did not significantly impact the oxidation kinetics. It is impossible to exclude the possibility that our samples may contain trace amounts of free FAD, but as clearly shown in Figure 1A, the FAD does appear to be protein-bound because it has the structured 443 and 470 nm absorption bands. Free FAD has a single broad absorption band at 450 nm. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis run with oxidized samples from the kinetic experiments shows no changes compared to fresh samples. DNA repair activity assays were also conducted with oxidized protein from the kinetic experiments with ~50% of the repair activity regained after removal of the oxidizing agent; the reduction of the FAD cofactor appeared to be unaltered from that of untreated protein. In the most extreme example, VcCRY1 that was in a reaction mixture at 5 °C for 80 h still had ~15% of full activity.

Binding studies using isothermal titration calorimetry were completed on VcCRY1 using the UV-p(dT)₁₀ substrate at 15 °C to see if any obvious differences exist in the substrate binding as the pH and oxidation state of the flavin cofactor are changed (see the Supporting Information). There was no detectable effect of oxidation state or pH on the $\Delta G^{\circ}_{\text{binding}}$ obtained. $\Delta G^{\circ}_{\text{binding}}$ was found to be –30 to –31 kJ/mol for all oxidation states at pH 6.40 and 7.96; these are similar to the values obtained for EcPL at the same temperature with the same substrate at pH 7.00.¹⁵

There does appear to be a significant difference measured for $\Delta H^{\circ}_{\text{binding}}$; at pH 6.40, it ranged from –12 ± 1 kJ/mol for the FADH[–] state to –13 ± 2 kJ/mol for the FADH[•] state and –15 ± 5 kJ/mol for the FAD state. At high pH, the enthalpy for the fully reduced state slightly decreased to –16 ± 6 kJ/mol while the enthalpy for the FAD state more sharply decreased to –24 ± 3 kJ/mol. No binding data were obtained for the FADH[•] state at high pH. The values obtained for EcPL with the same substrate but at pH 7.00 were different but also dependent on oxidation state: ΔH° values ranged from –27 kJ/mol for the PL FAD state to –40 kJ/mol for the PL FADH[–] state.¹⁵

To summarize the experimental data, the electrochemistry and pH-dependent behavior of VcCRY1, a single-strand CPD photolyase and CRY-DASH protein, are significantly different from those of EcPL, the best understood CPD photolyase. Formation of the FAD state is blocked in VcCRY1 when UV-damaged DNA is bound. No such effect was found with EcPL, although binding of substrate to EcPL does increase the reduction potential of the FADH[–]/FADH[•] couple by ~100 mV¹⁶ and protects the enzyme against degradation.¹⁷

Interpretation of the experimental data requires some understanding of the process required to oxidize FAD from the neutral FADH[•] in the protein. The O'Neil laboratory has contributed significantly to our understanding of this reaction in the PL and CRY families.^{18,19} Damiani, Yalloway, Lu et al. studied the FADH[•] states in PL from *Anacystis nidulans* (AnPL) and *Synechocystis* CRY-DASH.¹⁸ They concluded that the FADH[•] state in AnPL was kinetically stabilized by the transfer of a proton from the N5 atom in the isoalloxazine ring (structure in Figure 1A) as the rate-limiting step in the oxidation to FAD; they refer to that step as a kinetic barrier to FAD. This conclusion was based upon isotope studies showing that the oxidation of FAD requires proton-coupled electron transfer. The combination of a fast step to create the FADH[•]

state from FADH[–] followed by a slow step to form FAD results in a stable FADH[•] in PL; the CRY-DASH neutral radical was unstable because of FAD formation under the conditions (pH 7.00) they examined. They did identify two residues in the FAD binding pocket that they linked to the stability of the FADH[•] state: Trp392 and Gly389 in AnPL (and EcPL) compared to Trp398 and Asn395 in CRY-DASH (and VcCRY1). The residues are 7–13 Å from the N5 atom. On the basis of the large activation energy measured for the oxidation of VcCRY1 FAD in the presence of substrate, our results also suggest a large kinetic barrier.

Damiani, Nostedt, and O'Neill followed up with a second paper that expanded on this idea of a kinetic barrier.¹⁹ Using AnPL, they replaced the N5-proximal Asn386 with Asp, as found in the CRY family; they added an amino acid that could act as a proton shuttle from N5. They found formation of the FAD occurred much more rapidly than in the wild-type protein, similar to what is observed in the CRY proteins; the FAD will form when N5 is able to lose a proton to Asp. On the basis of these results, they proposed that the N5-proximal residue may serve to control the FADH[•] stability and, thus, the proton transfer, hydrogen bonding, and conformational changes that differentiate PL proteins from CRY proteins. The CRY-DASH proteins have Asn, not Asp, in the N5-proximal position, as shown with the published sequence alignment.¹⁹ We also expect Asn to be proximal to N5 for VcCRY1. From the literature, it appears plausible that the UV-p(dT)₁₀ molecule is acting to block proton transfer from the N5 position.

There are published crystal structures of *Arabidopsis thaliana* cryptochrome 3 (AtCry3), a CRY-DASH protein, with and without DNA bound (Protein Data Bank entries 2VTB and 2J4D).^{11,20} Klar, Pokorny, Moldt et al. clearly show Asn428 within 3.14 Å of the N5 atom of the isoalloxazine ring.²⁰ In our analysis of the two structures, there is very little change in the distance between Asn428 and N5 upon substrate binding, including any conveniently located water molecules. There are no clear structural changes to explain why proton transfer from N5 may be blocked by substrate binding. This may be evidence for a potential role that water molecules could be playing in the proton transfer step.

Additional hints of a possible explanation can be seen with the pre-Arrhenius factors (*A*) and activation energies. There are several parameters that may contribute to the theoretical pre-Arrhenius factor; it is a reflection of both the ensemble of ground state populations possible and the ensemble of pathways that may lead to the transition state.²¹ The ensemble populations and/or pathways may change with pH and temperature. In the Eyring treatment of transition state theory, *A* has a complicated relationship with ΔS^{\ddagger} , the entropic contribution to the formation of the activated complex; generally, a larger *A* would indicate a larger entropy change between the reactants and the activated complex. Using this simplistic interpretation, both the high- and low-pH reactions have high kinetic barriers to overcome, but the high-pH reaction occurs more readily because of a larger contribution from entropy.

The temperature dependence of the oxidation reaction is altered in the presence of damaged DNA substrate. The nonlinear behavior of the Arrhenius plot indicates that either the populations or the pathways of the reaction are changing with the temperature of the reaction mixture. The protein structure around the cofactor must be significantly altered

because of the presence of the substrate such that we measure nonlinear Arrhenius kinetics.

Our ITC data show that the protein–substrate interactions are altered with pH, especially in the FAD state where it appears that stronger interactions are formed based upon a more exothermic heat of binding. We also observed in earlier Raman studies¹² of VcCRY1 that hydrogen bonding around the cofactor changes with substrate binding. Therefore, it may be that the hydrogen bonding between the substrate and protein is substantially modified with the change in pH. In addition, because we are seeing large changes in the kinetics between pH 6.30 and 7.75, we may be seeing the direct involvement of water molecules in the proton transfer from the N5 group because few amino acids are expected to have pK_a values in that range.²² Further work is required to elucidate the changes observed with substrate binding.

In conclusion, the potential biological ramifications of these results are significant. We would anticipate the FADH[•] state to be destabilized for VcCRY1 *in vivo* because the published pH for *Vibrio cholerae* is 7.2.²³ Therefore, the FAD state should form readily in VcCRY1 in the absence of CPD lesions on DNA; in this case, the protein can act as a cog in the machine for the circadian clock control. In the presence of the CPD lesions, as expected at high light intensities, the cofactor will be trapped between the FADH[−] and FADH[•] states. Because FADH[•] readily photoreduces to form the FADH[−] state required for DNA repair, the protein will be able to repair the bound DNA in the presence of light. This hypothesis is an elegant explanation for the dual role of the CRY-DASH proteins observed *in vivo*.

■ ASSOCIATED CONTENT

■ Supporting Information

Materials and methods along with additional experimental results. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00307.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Chaves, I., Pokorny, R., Byrdin, M., Hoang, N., Ritz, T., Brettel, K., Essen, L.-O., van der Horst, G. T. J., Batschauer, A., and Ahmad, M. (2011) *Annu. Rev. Plant Biol.* 62, 335–364.
- (2) Sancar, A. (2003) *Chem. Rev.* 103, 2203–2237.
- (3) Bouly, J.-P., Schleicher, E., Dioisio-Sese, M., Vandenbussche, F., Van Der Straeten, D., Bakrim, N., Meier, S., Batschauer, A., Galland, P., Bittl, R., and Ahmad, M. (2007) *J. Biol. Chem.* 282, 9383–9391.
- (4) Müller, P., Bouly, J.-P., Hitomi, K., Baland, V., Getzoff, E. D., Ritz, T., and Brettel, K. (2014) *Sci. Rep.* 4, 5175–5186.
- (5) Ozturk, N., Selby, C. P., Zhong, D., and Sancar, A. (2014) *J. Biol. Chem.* 289, 4634–4642.
- (6) Fortunato, A. E., Annunziata, R., Jaubert, M., Bouly, J.-P., and Falciorato, A. (2015) *J. Plant Physiol.* 172, 42–54.
- (7) Facella, P., Lopez, L., Chiappetta, A., Bitonti, M. B., Giuliano, G., and Perrotta, G. (2006) *FEBS Lett.* 580, 4618–4624.
- (8) Heijde, M., Zabulon, G., Corellou, F., Ishikawa, T., Brazard, J., Usman, A., Sanchez, F., Plaza, P., Martin, M., Falciorato, A., Todo, T., Bouget, F.-Y., and Bowler, C. (2010) *Plant, Cell Environ.* 33, 1614–1626.
- (9) Biernat, M. A., Eker, A. P. M., van Oers, M. M., Vlak, J. M., van der Horst, G. T. J., and Chaves, I. (2012) *J. Biol. Rhythms* 27, 3–11.
- (10) Selby, C. P., and Sancar, A. (2006) *Proc. Natl. Acad. Sci. U.S.A.* 103, 17696–17700.
- (11) Pokorny, R., Klar, T., Hennecke, U., Carell, T., Batschauer, A., and Essen, L.-O. (2008) *Proc. Natl. Acad. Sci. U.S.A.* 105, 21023–21027.
- (12) Sokolowsky, K., Newton, M., Lucero, C., Wertheim, B., Freedman, J., Cortazar, F., Czocho, J., Schelvis, J. P. M., and Gindt, Y. M. (2010) *J. Phys. Chem. B* 114, 7121–7130.
- (13) Dutton, P. L. (1978) *Methods Enzymol.* 54, 411–435.
- (14) Zieba, A. A., Richardson, C., Lucero, C., Dieng, S. D., Gindt, Y. M., and Schelvis, J. P. M. (2011) *J. Am. Chem. Soc.* 133, 7824–7836.
- (15) Wilson, T. J., Crystal, M. A., Rohrbaugh, M. C., Sokolowsky, K. P., and Gindt, Y. M. (2011) *J. Phys. Chem. B* 115, 13746–13754.
- (16) Gindt, Y. M., Schelvis, J. P. M., Thoren, K. L., and Huang, T. H. (2005) *J. Am. Chem. Soc.* 127, 10472–10473.
- (17) Herriott, R. M. (1979) *Anal. Biochem.* 95, 77–81.
- (18) Damiani, M. J., Yalloway, G. N., Lu, J., McLeod, N. R., and O'Neill, M. A. (2009) *Biochemistry* 48, 11399–13411.
- (19) Damiani, M. J., Nostedt, J. J., and O'Neill, M. A. (2011) *J. Biol. Chem.* 286, 4382–4391.
- (20) Klar, T., Pokorny, R., Moldt, J., Batschauer, A., and Essen, L.-O. (2007) *J. Mol. Biol.* 366, 954–964.
- (21) Callender, R., and Dyer, R. B. (2015) *Acc. Chem. Res.* 48, 407–413.
- (22) Schönicke, A., Webb, B. A., Jacobson, M. P., and Barber, D. L. (2013) *Annu. Rev. Biophys.* 42, 289–314.
- (23) Sánchez, E., García, S., and Heredia, N. (2010) *Appl. Environ. Microbiol.* 76, 6888–6894.