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# Very Fast Product Release and Catalytic Turnover of DNA Photolyase

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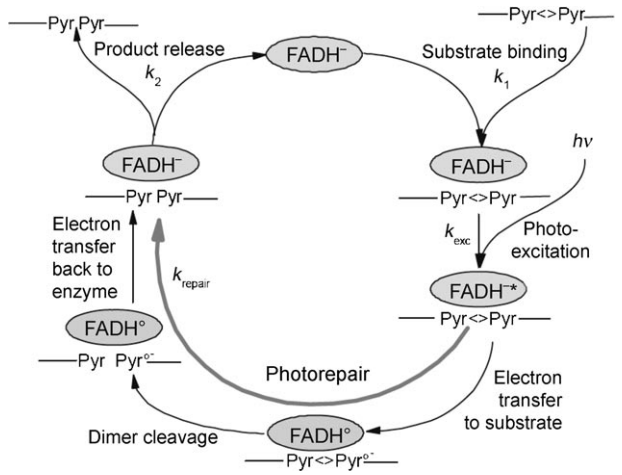
DNA photolyase is a flavoprotein that uses light to reverse some of the major UV-induced damage to DNA—cyclobutane pyrimidine dimers (CPD)—to the intact constituting bases, thus protecting the host organism from potentially harmful mutations.[1,2] Upon binding of photolyase to damaged DNA ($k_1$ in Scheme 1), the CPD is flipped out of the double helix




**Scheme 1.** Schematic representation of the catalytic cycle of DNA photolyase.

and enters into the binding pocket of the enzyme,[3] thus approaching the FAD cofactor (3.1 Å in CPD photolyase[4] and even 2.7 Å in the closely related (6–4) photolyase.[5]) Following excitation of FAD in its fully reduced state ($FADH^-$), an electron is transferred to the CPD, thereby initiating the splitting of the dimer, and subsequently returned to the flavin. The overall rate $k_{repair}$ of these photochemical repair steps is $\sim 10^9\ s^{-1}$.[6–8] Despite the fast repair reaction, the catalytic turnover number of photolyase under saturating continuous light has been reported to be only in the order of 0.1 to 1 $s^{-1}$;[9–11] this suggests that exchange of repaired DNA (product) for damaged DNA (substrate) is slow and rate limiting. While substrate binding was found to be rather fast ($k_1 \sim 10^6\ M^{-1}\ s^{-1}$ [12,13]), the rate of product release ($k_2$) has not been established as yet, and this step might well be rate limiting. Interestingly, in an X-ray diffraction experiment at 100 K, the restored pyrimidines remained in the binding pocket in close proximity to the flavin throughout data collection.[4] Here, we accessed product-for-substrate exchange directly with a time-resolved experiment based on the well-established[7,14,15] quenching of $FADH^-$ fluorescence by electron transfer to a T<>T–CPD present in the binding pocket. Surprisingly, following an intense repair flash, the flavin fluorescence did not recover immediately, but rather with a time constant of ~50 μs (at 10 °C). This observation suggests that the restored thymines act as electron acceptors and hence as fluorescence quenchers of nearby excited $FADH^-$; the hitherto unobserved 50 μs kinetics then reflect product release from the binding pocket and set an upper limit of $2\times10^4\ s^{-1}$ to the catalytic turnover number of photolyase. To verify this prediction, we re-examined the turnover number of photolyase under strong continuous laser light and a high substrate concentration. A rate of $260\ s^{-1}$ was observed, more than 100 times faster than previously reported.

The quenching of $FADH^-$ fluorescence by the CPD has been used for titration of binding processes[14] by applying steady-state fluorescence spectroscopy. Time-resolved fluorescence studies revealed that the fluorescence decay of $FADH^-$ accelerates from 1.4 ns in the absence of substrate to 160 ps when a CPD is bound.[15] Based on this difference in lifetime, we designed an experiment of the pump–probe principle: a strong actinic flash is applied to repair the substrate bound at the enzyme's active site, and a weak probe flash at variable time delay serves to read out the fluorescence intensity and kinetics as a measure for the occupation of the substrate binding pocket.

To avoid complications due to the antenna chromophore present in photolyases, we used an apophotolyase from *Anacystis nidulans* that was overexpressed in *E. coli* and is devoid of the 8-HDF antenna chromophore.[16] The substrate was a UV-irradiated $dT_{18}$ oligonucleotide that contained on average six randomly distributed CPDs (see the Supporting Information for details on enzyme and substrate preparation). Note that our substrate is heterogeneous with respect to the number and distribution of the CPDs per strand. Presence of this substrate quenched the steady-state fluorescence of $FADH^-$ in photolyase by 83% (Figure 1, inset).

Time-resolved $FADH^-$ fluorescence traces induced by a weak probe flash of 100 ps duration are presented in the main panel of Figure 1. In the absence of substrate (thick black trace), the fluorescence had a lifetime of 1.3 ns (thin black line; see Sup-

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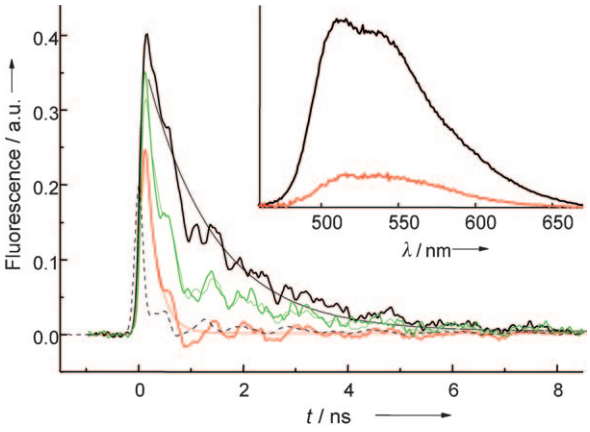


**Figure 1.** Substrate effects on the fluorescence of $FADH^-$ in DNA photolyase (13 μM). Main: fluorescence emission transients at 580 nm induced by a single 355 nm, 100 ps, 250 $\mu J\,cm^{-2}$ probe flash. Black: photolyase without substrate. Red: substrate with a total of 280 μM CPD added. Thin lines: mono-exponential fits. Thick green line: same sample as red, but with a 355 nm, 5 ns, 90 $mJ\,cm^{-2}$ repair flash fired 300 μs before the probe flash. Thin green line: weighted sum of the two other kinetics (45% black + 55% red). Dashed black line: instrument response to the scattered probe flash. Inset: Steady-state fluorescence emission spectra for the "black" and "red" samples from the main panel, excitation at 350 nm. All fluorescence curves have been corrected for a contribution from oxidized FAD (see the Supporting Information).

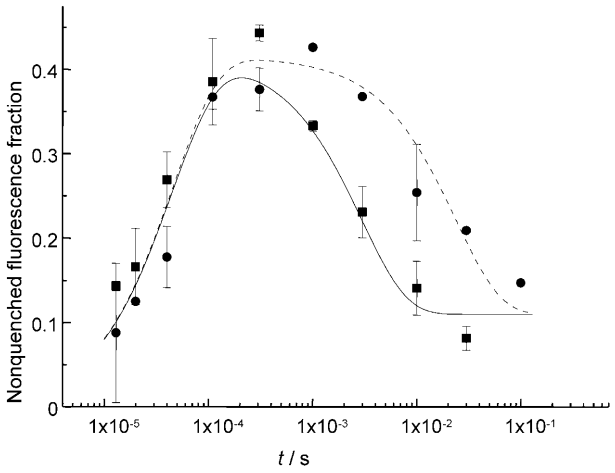


**Figure 2.** Kinetics of formation and disappearance of the nonquenched fluorescence fraction of DNA photolyase at time $t$ after a nearly saturating repair flash for two different substrate concentrations (–■–: 280, -----●-----: 35 μM CPD). Data points are derived from analysis of fluorescent transients as the green curve in Figure 1. Error bars represent statistical standard deviations from up to three independent measurements. Lines represent the best fit to the data points according to Equation (1) (see the text and Supporting Information for detailed procedures).

porting Information for a more detailed kinetic analysis of the fluorescence traces). The presence of the substrate (red traces) accelerated the fluorescence decay to ~0.2 ns, corresponding to ~85% fluorescence quenching. When a high-energy repair flash ("pump") was fired 300 μs in advance, the fluorescence kinetics induced by the probe flash (thick green trace; substrate present) was intermediate between the two other traces and could be well-described by a weighted sum of them: 45% black + 55% red (thin green trace). The bimodal kinetics suggest the coexistence of two fractions: photolyases with CPD present (fast fluorescence decay) and photolyases with no CPD in the binding pocket (slow decay).

Fluorescence traces measured at various delays after the repair flash were analyzed (see the Supporting Information) for the fraction of photolyases behaving as the sample without CPD ("nonquenched", black trace in Figure 1). The results for two different substrate concentrations are presented in Figure 2. Both data sets show an increase in the nonquenched fraction over ~50 μs to a maximal value of ~0.45 and a subsequent decline on the millisecond scale that is accelerated at higher substrate concentrations. While the declines depend on substrate concentration and can be attributed to rebinding of CPD with a rate constant of the order of $10^6\,M^{-1}s^{-1}$ as reported,[1,12,13] the increase in the nonquenched fraction on a 100 μs timescale appears in contradiction to the reported[6–8] fast (~1 ns) repair of the CPD that should delete the fluorescence quencher and result in a quasi immediate onset of the nonquenched fluorescence.

We therefore suggest that restored thymine, as long as present in the binding pocket, functions as electron acceptor and hence as fluorescence quencher of excited $FADH^-$. Studies on 1,3-dimethyl thymine analogues suggest that the reduction potential of thymine is slightly higher than that of the T<>T dimer,[17] so electron transfer from excited $FADH^-$ to thymine should be at least as thermodynamically feasible as that to the CPD. Moreover, Carell and co-workers observed that thymine can function as a stepping stone for the transfer of electrons injected into DNA by a covalently linked excited $FADH^-$.[18,19] The nonquenched fraction in Figure 2 should hence represent the fraction of enzyme with a binding pocket that contains neither CPD nor repaired thymine.

For quantitative analysis of product release after repair and the following rebinding of substrate, we considered the simplified reaction Scheme 2 (indices of $k$ correspond to Scheme 1):

$$EP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P \qquad E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

**Scheme 2.** Simplified reaction scheme.

Here E denotes enzyme, P product and S substrate. The reverse reactions ($k_{-2}$ and $k_{-1}$) were neglected because of the high specificity of photolyase for the substrate,[1] which was present in high excess. To account for a not fully saturating repair flash and for a repair quantum yield lower than 100%, it was assumed that repair occurred only in a fraction $\beta$ of the enzyme–substrate complexes. Relevant concentrations at time zero (almost immediately after the pump flash, when the photorepair is just completed) were set to $[EP]_{t=0}=\beta[E]_{tot}$, $[S]_{t=0}=[S]_{tot}-[E]_{tot}$, $[E]_{t=0}=0$. $[E]_{tot}$ and $[S]_{tot}$ correspond to the total amounts of added enzyme and CPDs, respectively, and $[S]_{tot} \gg$

  

$[E]_{tot}$ in our experiments. Neglecting the slight variation of [S] during the reaction, the free enzyme fraction is then given by the expression for the intermediate in a sequential reaction:

$$\frac{[E](t)}{[E]_{tot}} = \beta \frac{k_2}{k_2 - k_1'} (\exp(-k_1' t) - \exp(-k_2 t)) \quad (1)$$

Here $k_1'$ is given by $k_1' = k_1([S]_{tot} - [E]_{tot})$. To account for the heterogeneity of substrate binding,[12,20,21] it was admitted that a fraction $\gamma$ of the enzymes binds substrate more slowly than the timescale of our experiment ($k_1$ fixed to zero for this fraction).

A simultaneous least-squares fit (solid lines) to both data sets in Figure 2 yielded $k_1 = 1.2 \times 10^6\ M^{-1} s^{-1}$, $k_2 = 1.8 \times 10^4\ s^{-1}$, $\beta = 0.41$ and $\gamma = 0.28$. The found substrate binding rate constant $k_1$ is close to literature values.[1,12,13] However, our product release rate, $k_2$, is more than four orders of magnitude faster than what we had expected based on the low catalytic turnover numbers (0.1 to 1 $s^{-1}$) reported in the literature[9–11] for saturating illumination (see above).

Note, our evaluation of the fluorescence data was based on the plausible, but unproven, assumption that restored thymine still present in the binding pocket is reduced by excited $FADH^-$ at a rate comparable to that of the CPD. Direct evidence for thymine reduction by excited $FADH^-$ might be obtained by double-flash transient-absorption experiments in the UV region. Unfortunately, as the ultrafast pump–probe technique relies on accumulation of an excessively large number of experiments,[6] each on a sample containing unrepaired CPD, its application seems practically impossible so far (by contrast, single shots were sufficient to resolve the fluorescence kinetics in the present approach). Therefore, we searched for further support for fast product release by re-examining the catalytic turnover number under the strongest continuous illumination available in our laboratory.

We focused the near-UV lines (350 and 363.8 nm) of a continuous-wave argon ion laser on a pinhole of 0.8 mm diameter and placed a sample containing ~10 μM photolyase and substrate with a total of ~250 μM CPD behind the pinhole. Exposition times were varied between 200 μs and 40 ms, and, for each exposition time, the number $n$ of CPDs repaired per reduced photolyase present in the illuminated volume was determined from the absorption increase at 265 nm (Figure 3). For short expositions, $n$ increased steeply with exposition time until close to one CPD was repaired per photolyase (-----). Then the increase slowed and remained linear up to 10 ms exposition (——). For longer expositions (not shown), substrate depletion compromised the further increase of $n$. The fast initial rise (slope ~$10^3$ $s^{-1}$, dashed) reflects repair of the CPD already bound prior to illumination; this rate corresponds to the excitation rate ($k_{exc}$ in Scheme 1), corrected for the repair quantum yield. Once the initially bound CPD is repaired, substrate exchange becomes necessary for further repair. The slope of the repair curve between ~1 and 10 ms exposure time corresponds to a multiple turnover repair number of 260 $s^{-1}$ (solid line in Figure 3), basically limited by substrate binding ($k_1$ [CPD] = 300 $s^{-1}$ with $k_1$ determined from Figure 2). These observations prove that the product release rate, $k_2$, is faster than 260 $s^{-1}$ and strongly support our above interpretation of the fluorescence data.

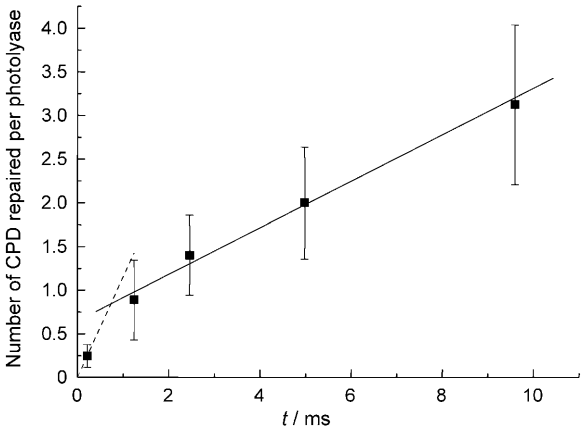


**Figure 3.** Extent of CPD repair due to strong light pulses of varying duration $t$. Samples containing 10 μM reduced photolyase and substrate with a total of 250 μM CPD were exposed for the indicated times to ~120 W $cm^{-2}$ near-UV irradiation (350 and 363.8 nm lines of an $Ar^+$ laser). –■–: repair as quantified from the absorbance increase around 265 nm. Error bars represent standard deviations from multiple experiments. ——: linear fit to the data corresponding to the multiple-turnover regime. The slope of the dashed line represents the initial repair rate corresponding to the single-turnover regime.

In summary, we have discovered that the quenching of $FADH^-$ fluorescence in DNA photolyase persists for ~50 μs after photorepair of the CPD. We assign this quenching to electron transfer from excited $FADH^-$ to restored pyrimidine bases still present in the substrate binding pocket, and conclude that the release of this reaction product occurs in ~50 μs, that is, four orders of magnitude faster than expected from published catalytic turnover numbers of, at best, 1 $s^{-1}$. In support of this suggestion, we were able to achieve a multiple turnover number as high as 260 $s^{-1}$ by using strong illumination from a laser source and a high, but still rate-limiting, substrate concentration. Previous turnover measurements might have underestimated the light intensity that is necessary to saturate the catalytic turnover of photolyase. Even if physiological light conditions do not allow the turnover number found here to be achieved in vivo, this knowledge is crucial for in vitro experiments with repetitive laser excitation.

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