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ARTICLE *in* BIOCHIMICA ET BIOPHYSICA ACTA · OCTOBER 2006

Impact Factor: 4.66 · DOI: 10.1016/j.bbapap.2006.06.016 · Source: PubMed

Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1764 (2006) 1454–1461
BIOCHIMICA ET BIOPHYSICA ACTA
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Reversible resolution of flavin and pterin cofactors of His-tagged *Escherichia coli* DNA photolyase

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Received 25 January 2006; received in revised form 5 June 2006; accepted 13 June 2006
Available online 28 July 2006

Abstract

Escherichia coli photolyase catalyzes the repair of cyclobutane pyrimidine dimers (CPD) in DNA under near UV/blue-light irradiation. The enzyme contains flavin adenine dinucleotide (FAD) and methenyltetrahydrofolate (MTHF) as noncovalently bound light sensing cofactors. To study the apoprotein–chromophore interactions we developed a new procedure to prepare apo-photolyase. MTHF-free photolyase was obtained by binding the C-terminal His-tagged holoenzyme to a metal-affinity column at neutral pH and washing the column with deionized water. Under these conditions the flavin remains bound and the defolated enzyme can be released from the column with 0.5 M imidazole pH 7.2. The MTHF-free protein was still capable of DNA repair, showing 70% activity of native enzyme. Fluorescence polarization experiments confirmed that MTHF binding is weakened at low ionic strength. Apo-photolyase was obtained by treating the His-tagged holoenzyme with 0.5 M imidazole pH 10.0. The apo-photolyase thus obtained was highly reconstitutable and bound nearly stoichiometric amounts of FAD_{ox}. Photolyase reconstituted with FAD_{ox} had about 34% activity of native enzyme, which increased to 83% when FAD_{ox} was reduced to $FADH^-$. Reconstitution kinetics performed at 20 °C showed that apo-photolyase associates with $FADH^-$ much faster (k_{obs} ~3000 M^{-1} s^{-1}) than with FAD_{ox} (k_{obs}=34 M^{-1} s^{-1}). The dissociation constant of the photolyase–FAD_{ox} complex is about 2.3 μM and that of E-$FADH^-$ is not higher than 20 nM (pH 7.2).

Keywords: Apoflavoprotein; Cofactor binding; Flavin; IMAC; Photolyase; Pterin; Reconstitution

1. Introduction

UV irradiation can induce DNA damage by stimulating the formation of cyclobutane pyrimidine dimers (CPD). The lesions are harmful to the organism. *E. coli* photolyase (EC 4.1.99.3) can catalyze the repair of CPD adducts under 350–450 nm light irradiation. The enzyme is a monomeric protein of 471 amino acids and contains two noncovalently bound chromophore cofactors [1]. One cofactor is flavin adenine dinucleotide (FAD) [2]. This molecule is bound in the core of the protein in a U-shaped conformation [3] and is essential both for specifically binding to damaged DNA and for catalysis [4–8]. The active form of DNA photolyase, both *in vivo* and *in vitro*, requires anionic two-electron reduced FAD ($FADH^-$) [4,5]. However, in freshly isolated enzyme the flavin is present in the neutral semiquinone ($FADH^•$) state [2]. During storage, this blue form of the flavin is slowly oxidized to FAD_{ox} and the enzyme becomes green to yellow [6,7]. The catalytically inert radical and oxidized forms of DNA photolyase can be reduced by dithionite [2] or by illumination in the presence of dithiothreitol [9] to restore activity.

The other cofactor is 5,10-methenyltetrahydropterolypoly-glutamate (MTHF) [10,11]. This pterin derivative is located at the surface of the protein with the pterin ring inserting into a cleft and acts as an antenna molecule by transferring energy to the flavin during the photoreaction [12]. MTHF is not crucial for the structure and the defolated enzyme is still catalytic [3,4]. MTHF is usually present in the purified enzyme in substoichiometric amounts and containing three to six glutamates in its tail [10]. It has been proposed that salt bridges between the

Abbreviations: FAD, flavin adenine dinucleotide; FAD_{ox}, oxidized FAD; $FADH^•$, neutral FAD radical; $FADH^-$, reduced anionic FAD; MTHF, methenyltetrahydrofolate; CPD, cyclobutane pyrimidine dimer; DTT, dithiothreitol

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doi:10.1016/j.bbapap.2006.06.016

polyglutamate tail of MTHF and protein surface residues increase the affinity of the photolyase–MTHF complex [1]. However, recent mutagenesis studies show that some residues in the binding cleft, such as Glu 109, are crucial for MTHF binding [13,14].

Much insight into the mechanism of DNA photolyase has been gained from reconstitution experiments with natural and artificial cofactors [15,16]. A prerequisite for such studies is that the cofactors are released under mild conditions, preventing the loss of protein and reconstitutable activity [17]. Both flavin and pterin cofactors can be released from *E. coli* photolyase by immobilization of the enzyme on phenyl-Sepharose and washing the column with pH 3.5 buffer containing high concentrations of ammonium sulfate and KBr [15]. Recovery of the apoprotein is then achieved by eluting the column with neutral buffer containing 50% ethylene glycol. Although this procedure is relatively mild and applicable for a number of flavoproteins [17,18], it does not allow the removal of one of the cofactors of DNA photolyase specifically.

Recently, a His-tag based immobilization method for the preparation and reconstitution of apoflavoproteins was described [19]. This prompted us to investigate whether this strategy could be applied for the reversible resolution of the flavin and pterin cofactors of *E. coli* photolyase. Here we show that the flavin and pterin cofactors can be reversibly removed by treating the C-terminal His-tagged enzyme with alkaline imidazole. Pterin-free holoenzyme was obtained by washing the immobilized enzyme with deionized water. This latter procedure can be performed at large scale and provides an attractive alternative to other methods such as sodium borohydride reduction [4] and photodecomposition [20]. Some physical properties of the various forms of photolyase and the kinetics of holoenzyme reconstitution are also described.

2. Materials and methods

2.1. Plasmid and reagents

The *phr*(*N*+*X*) plasmid was constructed by inserting the *E. coli phr*B gene into pET22b(+) (Novagen) between the restriction sites of *Nde*I and *Xho*I. This procedure added a six-histadine-tag coding sequence to the photolyase gene [21]. Lysis buffer contained 10% glycerol, 100 mM NaCl, 50 mM Tris–HCl, pH 7.2. Chelating Sepharose Fast Flow and Sephadex gel filtration resins were from Pharmacia. Single strand DNA cellulose was purchased from Sigma.

Chelating sepharose Fast Flow was activated by loading the resin with Ni^{2+}. Starting buffer of the chelating column contained 50 mM Tris–HCl, 10% glycerol, 500 mM NaCl, 50 mM imidazole, pH 7.2. Elution buffer contained 50 mM Tris–HCl, 10% glycerol, 100 mM NaCl, 500 mM imidazole, pH 7.2. Buffer A, B, C contained 50 mM, 500 mM and 10 mM NaCl, respectively, in 10% glycerol, 1 mM EDTA, 50 mM Tris–HCl, pH 7.2.

FAD disodium salt (Sigma) was dissolved to 0.1 M, and stored at −20 °C. Oligothymidylates [oligo $(dT)_{16}$] (Sangon, Shanghai) were dissolved in distilled water to 100 μM and irradiated under a 300 W high-pressure Hg lamp to generate pyrimidine dimers [21]. Stock solutions of sodium dithionite in oxygen free water (flushed with argon) were prepared immediately before use.

2.2. Purification of C-terminal His(6)-tagged photolyase

The *phr*(*N*+*X*) plasmid was transferred into competent cells of *E. coli* strain BL21(DE3). The culture conditions were as previously reported [21]. Induced cells of 10 g wet weight containing overexpressed photolyase were collected by centrifugation, resuspended in 35 ml lysis buffer and then disrupted by French Press. After centrifugation the supernatant was loaded onto a Ni^{2+} chelating sepharose Fast Flow column (30×15 mm) and incubated for 30 min. Then the column was washed with starting buffer and the photolyase was desorbed with elution buffer. Subsequently, the imidazole was removed by buffer exchange on Sephadex G15. Photolyase purified in this way was about 95% pure. Further purification was performed with a DNA cellulose column (30×15 mm). After binding in buffer C and washing the column with 2 volumes of buffer C, the enzyme was eluted with 1.5 volumes of buffer B. Generally, the buffer was changed to buffer A by Sephadex G15 chromatography. The two-step purification of His-tagged *E. coli* photolyase gives about 80% yield with a purity of more than 98%.

2.3. Apoprotein preparation

All experiments were performed at 4–8 °C. Apo-photolyase was prepared by incubating 20 ml of 10 μM enzyme for 4–12 h in 50 mM Tris–HCl, 10% glycerol, 50 mM NaCl, 500 mM imidazole, pH 10.0. FAD, MTHF (decomposed products) and imidazole were then removed with a Sephadex G50 column (200×15 mm) running in buffer A. The apoprotein could also be prepared by first binding the holoenzyme to the immobilized nickel affinity column and then washing the column with 50 mM Tris–HCl, 10% glycerol, 50 mM NaCl, 500 mM imidazole, pH 10.

To prepare photolyase containing FAD_{ox}, 10 μM apo-photolyase was incubated with 200 μM FAD overnight. The enzyme was then loaded onto a DNA cellulose column (30×15 mm), washing off excess FAD with buffer C, and then eluting the FAD_{ox} reconstituted enzyme with buffer B. If needed, the buffer was changed to buffer A by Sephadex G15 chromatography. Photolyase containing $FADH^-$ was prepared by adding 3 mM sodium dithionite to photolyase containing FAD_{ox} in buffer A.

To prepare defolated photolyase, 15 ml of 20 μM holoenzyme was bound to the immobilized nickel affinity column (30×15 mm) in starting buffer. The affinity matrix was then washed with 30 column volumes deionized water before eluting the enzyme with elution buffer pH 7.2. Subsequently, imidazole was removed by buffer exchange on Sephadex G15.

2.4. Activity determinations

The activity determinations of different forms of photolyase were performed according to an early report [22] with minor modifications. Assays were performed in aerobic conditions (except for fully reduced form) in buffer A, containing 1 mM DTT as external electron donor. The assay volume was 400 μl. 2 μM enzyme was incubated in a 700 μl quartz cuvette with 4 μM $(dT)_{16}$ containing about 5.5 dimers per molecule. The black light lamp of 15 W with wavelength major in 365 nm was used as irradiation light. The reaction mixture was incubated in the dark for 10 min, and then irradiated with a lamp distance of about 4 cm. The experiments were performed at 20 °C as controlled by a central air conditioner and the activity was determined from the increase of absorbance at 268 nm.

2.5. Absorption spectroscopy

Absorption spectra were recorded from 200 to 700 nm on a Beckman DU640 spectrophotometer. A 300 μl quartz cuvette was used and the temperature was held at 15 °C. The following molar absorption coefficients were used to determine the concentrations of the various protein species: enzyme-bound $FADH^\bullet$ ε_{580}=4800 M^{-1} cm^{-1}, enzyme-bound FAD_{ox} ε_{443}=11,200 M^{-1} cm^{-1} [15], apo-photolyase ε_{280}=103,630 M^{-1} cm^{-1} (by online prediction program PROTPARAM, www.expasy.org/tools/protparam.html). The molar absorption coefficient of enzyme-bound FAD_{ox} was assumed to be the same as that of free FAD_{ox}, ε_{280}=21,200 M^{-1} cm^{-1} [23]. This value was used to estimate the binding rate of FAD_{ox} to apo-photolyase.

2.6. Steady-state fluorescence polarization

Steady-state fluorescence polarization measurements were made with a PerkinElmer LS 55 fluorescence spectrophotometer. The fluorescence-polarization

of protein-bound MTHF was determined from freshly purified enzyme which contains $FADH^{\bullet}$ and about 60% MTHF. The experiments were performed in buffer A, buffer B, and buffer C, respectively. The excitation wavelength was fixed at 360 nm. The emission wavelength was 470 nm for MTHF [15].

2.7. Determination of the K_D of apo-photolyase to FAD_{ox}

Fluorescence measurements were made on an Aminco-Bowman series 2 Luminescence Spectrometer (Thermo electron). The excitation wavelength was set at 443 nm and the emission wavelength was 520 nm.

The K_D of the complex between apo-photolyase and FAD_{ox} was derived from the equation

$$I/I_f = I_b/I_f + \frac{-K_D + \sqrt{K_D^2 + 4k[E_0]}}{2[E_0]}(1 - I_b/I_f) \quad (1)$$

where I/I_f is the ratio of the fluorescence intensity of the enzyme solution (fluorescence of FAD_{ox} bound to the enzyme plus fluorescence of free FAD_{ox}) and free FAD_{ox}, and I_b/I_f is the ratio of the fluorescence intensity of protein-bound and free FAD_{ox}. $[E_0]$ is the concentration of the enzyme solution. I/I_f was obtained from the E-FAD_{ox} solution of various concentrations after at least 2 days equilibrating at 4 °C, and then adding 0.2% SDS to the solution to release FAD_{ox}. I_b/I_f and K_D were obtained by the Levenberg–Marquardt non-linear fitting method.

2.8. Kinetics of reconstitution

Kinetics of reconstitution was studied by time tracing fluorescence spectra, recorded on the Aminco-Bowman Luminescence Spectrometer. For reconstitution of the apoprotein–FAD_{ox} complex, FAD_{ox} of a series of concentrations, 20 μM, 10 μM, 5 μM and 2 μM, were added to 2 μM apo-photolyase, respectively. The excitation wavelength was at 443 nm and the emission maximum was at 520 nm. A blank experiment with 20 μM free FAD_{ox} in buffer A showed that under these conditions and during the entire experiment no detectable photoreduction occurred. Reconstitution of the apoprotein–$FADH^-$ complex was studied under strictly anaerobic conditions. To that end, samples of apo-photolyase and water to prepare sodium dithionite and $FADH^-$ solutions were flushed with argon for at least 10 min. The apo-photolyase solution was then added to a quartz cuvette and the surface sealed with mineral oil. $FADH^-$ solution was prepared from FAD_{ox} by dithionite reduction as confirmed by UV-vis spectral scanning. In the reconstitution reactions, 10 μM, 5 μM and 2.5 μM $FADH^-$, were added to equimolar concentrations of apo-photolyase and the tracings were started immediately. The excitation wavelength was at 360 nm, the emission maximum at 505 nm. All experiments were performed at 20 °C.

2.9. Fluorescence titration of apo-photolyase with $FADH^-$

Titration of apo-photolyase with $FADH^-$ was also performed on the Aminco-Bowman Luminescence Spectrometer. Anaerobic conditions were generated as described above. A portion of 0.9 μM apo-photolyase was titrated at 20 °C with $FADH^-$. The excitation wavelength was at 360 nm, the emission maximum at 505 nm, with slits set to 4 nm.

3. Results

3.1. Preparation and spectral properties of apo- and defolated photolyase

Due to the FAD and MTHF chromophores, photolyase displays distinguishable absorption spectral features in different redox states [7,15]. Fig. 1A shows that freshly purified His-tagged photolyase contains the neutral (blue) form of $FADH^{\bullet}$, which displays typical long-wavelength absorption maxima around 500 and 600 nm. The MTHF cofactor is responsible for the absorption peak around 380 nm. During storage under aerobic conditions, the FAD cofactor is slowly converted into the oxidized form, as evidenced from the increase in absorbance around 450 nm (Fig. 1A). The isosbestic point observed at 482 nm is similar to early reports [24,25].

Although the FAD cofactor becomes oxidized under aerobic conditions, considerable amounts of $FADH^{\bullet}$ remain

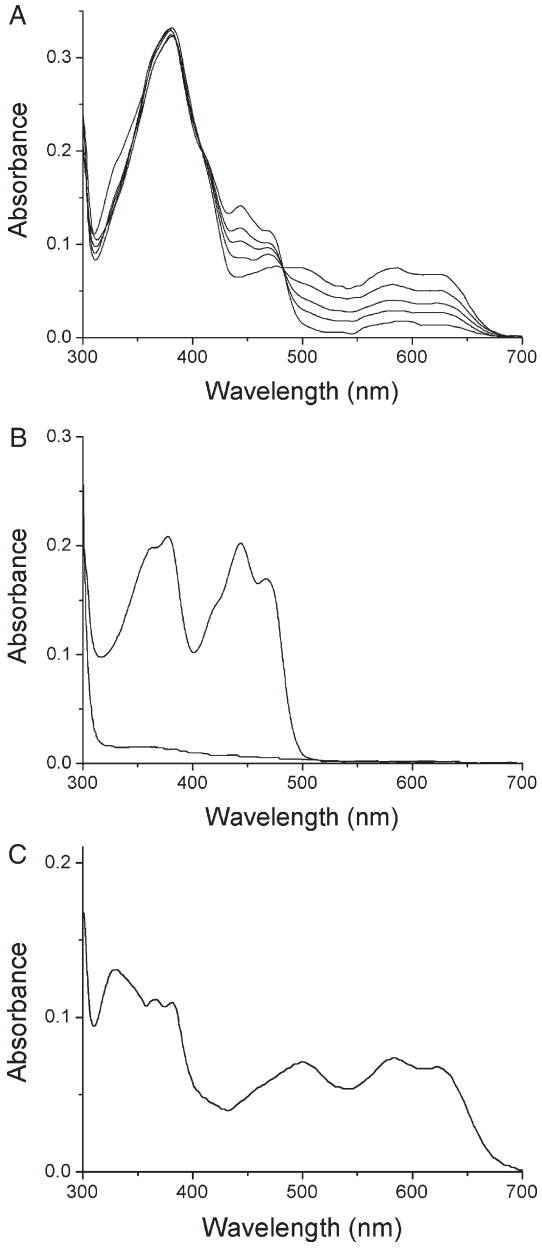

Fig. 1. Absorption spectra of various forms of His-tagged *E. coli* DNA photolyase. (A) Progressive changes of native photolyase (in buffer A) during storage at 4 °C in dark. Spectra were monitored with intervals of 2 days. The absorption decreased at long wavelength and increased around 443 nm, which is indicative for the oxidation of protein-bound $FADH^{\bullet}$. An isosbestic point is observed at 482 nm. (B) Spectra of apo-photolyase prepared by imidazole incubation and FAD_{ox} reconstituted enzyme. The apo-photolyase (bottom) has no pronounced absorption in 300–700 nm range. The reconstituted enzyme (top) has absorption bands at 378 nm and 443 nm with resolved shoulders at 360 nm, 420 nm and 467 nm. (C) Spectrum of defolated photolyase prepared by deionized water treatment.

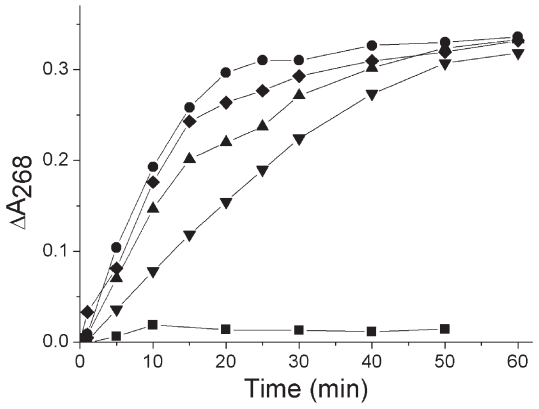

Fig. 2. Activity of various forms of His-tagged *E. coli* photolyase. Fresh radical form photolyase (MTHF plus FADH•) (●); defolated photolyase (FADH•) (▲); reconstituted enzyme (FAD_{ox}) (▼); reconstituted reduced photolyase ($FADH^-$) (◆); apoprotein (■).

present in DNA photolyase even after storing the enzyme for a long time [6]. To get fully oxidized photolyase, an optional method is to reconstitute apo-photolyase by FAD_{ox} [15,26]. Here we found that apo-photolyase can readily be obtained by incubating the holoenzyme for 4–12 h with 0.5 M imidazole pH 10.0 and subsequent Sephadex G-50 gel filtration. The apoprotein prepared in this way does not show any pronounced absorption from 300 nm to 700 nm indicating that both chromophore cofactors are removed. The presence of imidazole in the incubation buffer appeared to be essential because a similar treatment of the holoenzyme with 200 mM Tris–HCl pH 10.0, 50 mM NaCl, either immobilized to Ni–NTA Sepharose or free in solution, did not result in high yields of apoenzyme.

Reflavinylation of apo-photolyase is easily achieved by incubating the apoenzyme with excess FAD. The reconstituted apoprotein–FAD_{ox} complex shows absorption maxima at 378 nm and 443 nm with resolved shoulders at 360 nm, 420 nm and 467 nm (Fig. 1B). From the molar absorption coefficients of free FAD_{ox} ($\varepsilon_{443}=11.2\ mM^{-1}\ cm^{-1}$, $\varepsilon_{280}=21.2\ mM^{-1}\ cm^{-1}$) and the molar absorption coefficient of apo-photolyase ($\varepsilon_{280}=103.6\ mM^{-1}\ cm^{-1}$), it is estimated that about 0.95 mole of FAD_{ox} is bound per mole apoprotein.

His-tagged apo-photolyase could also be prepared by first binding the holoenzyme to Ni-NTA Sepharose and then washing the column with 50 mM Tris–HCl, 10% glycerol, 50 mM NaCl, 500 mM imidazole, pH 10.0. However, for obtaining apoenzyme with negligible residual activity it is essential to leave the eluted protein for at least 2 h in the basic imidazole solution.

Defolated photolyase was readily prepared by binding the His-tagged enzyme to Ni-NTA Sepharose and washing the column with deionized water. This method of MTHF removal is simple but efficient, and the FAD cofactor is not affected. Fig. 1C shows the absorption spectrum of defolated photolyase which only contains FADH•. This spectrum is highly similar to that of the defolated enzyme as prepared by photodecomposition [4] or sodium borohydride reduction [20].

3.2. Catalytic properties

To evaluate the new method of apoprotein preparation, activity assays were performed with the various photolyase forms. Assays were performed in aerobic condition, except for fully reduced form, which was in anaerobic condition to insure its redox state. Fig. 2 shows that freshly prepared holoenzyme (MTHF plus FADH•) has the highest activity ($0.53\pm0.05\ min^{-1}$, considered as 100% activity). After MTHF cofactor removal, the activity of the enzyme (E-FADH•) decreases to 70% ($0.37\pm0.02\ min^{-1}$). The reconstituted oxidized photolyase (E-FAD_{ox}) shows the lowest activity (34%, $0.18\pm0.01\ min^{-1}$). However, after dithionite reduction (E-$FADH^-$), the activity (83%, $0.44\pm0.02\ min^{-1}$) is higher than that of defolated photolyase. These results are consistent with an earlier report in values [15], except for the reduced form of the reconstituted enzyme which was reported to be only 47% active compared to native holoenzyme.

3.3. Fluorescence-polarization studies

The theory of fluorescence polarization was first described by Perrin in 1926 [27]. Small molecules rotate quickly during the excited state, and upon emission, have low polarization values. Large molecules rotate much slower during the excited state, and therefore have high polarization values.

We studied MTHF cofactor binding to apo-photolyase by fluorescence polarization. Fig. 3 shows that the fluorescence polarization of the enzyme-MTHF complex decreased with lowering the enzyme concentration and in dependence of the ionic strength of the buffer. High salt concentrations increased the fluorescence polarization. Because holoenzyme that contains flavin cofactor is in the monomeric state [1], the increase of fluorescence polarization is most probably due to stronger binding of MTHF and not due to protein oligomerization. This is in accordance with our finding that the MTHF cofactor is released from the photolyase by treating the immobilized enzyme with deionized water.

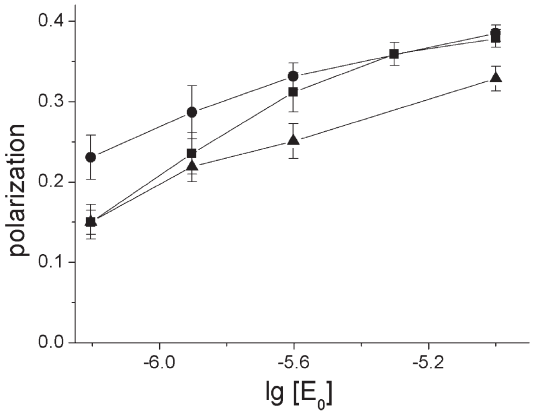

Fig. 3. Fluorescence polarization properties of MTHF cofactor of His-tagged *E. coli* photolyase in fresh radical form (containing about 60% MTHF) in buffer A (■), buffer B (●) and buffer C (▲), respectively.

3.4. Determination of the K_D of the complex between apo-photolyase and FAD_{ox}

To determine the K_D value of E-FAD_{ox}, we measured the fluorescence intensities of solutions of 12.6 μM, 7.7 μM, 3.9 μM, 1.9 μM and 1 μM E-FAD_{ox}. The intensities of the solution, after adding 0.2% SDS to release free FAD_{ox}, were also measured. By plotting I/I_f versus $[E_0]$ (Fig. 4) and fitting the data according to Eq. (1) values of $I_b/I_f=0.60\pm0.03$ μM and $K_D=2.3\pm0.9$ μM were estimated.

3.5. Reconstitution kinetics

To study the kinetics of FAD cofactor binding to apo-photolyase, we measured the rate of holoenzyme reconstitution by time tracing fluorescence experiments. Portions of 2 μM apo-photolyase (E) were mixed with 20 μM, 10 μM, 5 μM and 2 μM FAD_{ox}, respectively and the fluorescence intensity decrease was recorded with time (Fig. 5). The initial slopes obtained by linear fit were used to calculate the association rate constant (k_1):

$$\frac{d[\mathrm{E{-}FAD_{ox}}]}{dt} = k_1[\mathrm{E}][\mathrm{FAD_{ox}}] - k_2[\mathrm{E{-}FAD_{ox}}] \quad (2)$$

k_2[E-FAD_{ox}] was ignored for being negligible initially and [E] and [FAD_{ox}] were considered nearly constant. From the value of I_b/I_f of 0.60, we obtained a value of 34.4 ± 1.8 M^{-1} s^{-1} for k_1 from all above concentrations. From the values of K_D of 2.3 μM and k_1 of 34.4 M^{-1} s^{-1}, the dissociation rate constant k_2 was estimated to be about 7.9×10^{-5} s^{-1}.

We also measured the association rate of apo-photolyase and $FADH^-$. For this we made use of the fact that $FADH^-$ becomes fluorescent when binding to photolyase [4,28]. When equimolar amounts of $FADH^-$ were respectively added to 10 μM, 5 μM and 2.5 μM apo-photolyase under strictly anaerobic conditions, the associations of $FADH^-$ to apo-photolyase were completed within about 10 min (Fig. 6). Because high k_1 and low k_2 values

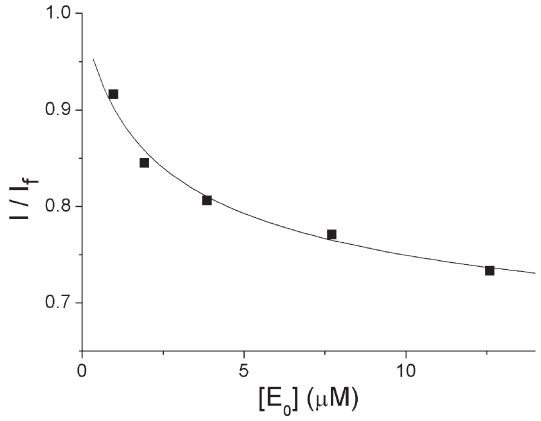

Fig. 4. Determination of the K_D of apo-photolyase to FAD_{ox} by the ratios of the fluorescence intensity (I/I_f) of the E-FAD_{ox} form of photolyase and free FAD_{ox}. Concentrations ($[E_0]$) used were 12.6 μM, 7.7 μM, 3.9 μM, 1.9 μM and 1 μM, respectively. The curve was obtained by the Levenberg–Marquardt non-linear fitting method to get the K_D.

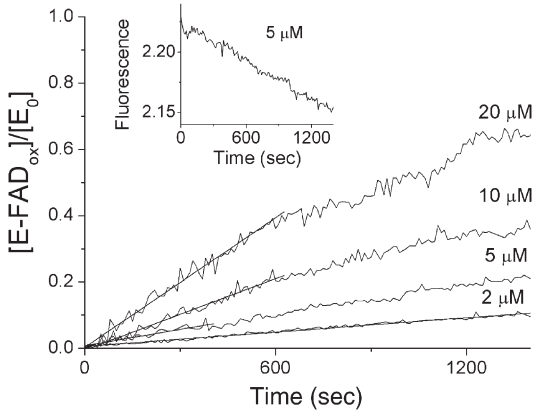

Fig. 5. Kinetics of reconstitution of apo-photolyase with FAD_{ox}. The reactions were of 2 μM apo-photolyase with 20 μM, 10 μM, 5 μM and 2 μM FAD_{ox}. The decreases in fluorescence intensity were normalized to the reconstituted ratios ([E-FAD_{ox}]/$[E_0]$). Initial slopes were obtained by linear fitting to calculate the k_1. The insert shows the decrease in fluorescence intensity with the time of the reaction of 2 μM apo-photolyase with 5 μM FAD_{ox}.

were estimated, k_2[E-$FADH^-$] was ignored. Under these conditions, [E] equals [$FADH^-$] and the association rate constant can be derived from:

$$k_1 t = \frac{1}{[E_0] - [\mathrm{E{-}FADH^-}]} - \frac{1}{[E_0]} \quad (3)$$

where $[E_0]$ and [E-$FADH^-$] represent the initial concentration of apo-photolyase and the concentration of formed reduced holoenzyme, respectively. By non-linear fitting, the association rate constants k_1 for the formation of the reduced holoenzyme were determined to be $3.05\pm0.15\times10^3$ M^{-1} s^{-1}, 4.57±

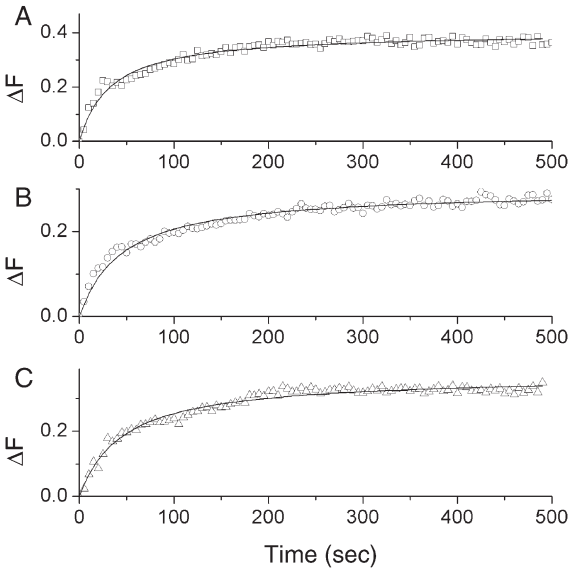

Fig. 6. Kinetics of reconstitution of apo-photolyase with $FADH^-$. Flavin fluorescence increases (ΔF) were upon reactions of: (A) 10 μM apo-photolyase with 10 μM $FADH^-$ (□); (B) 5 μM apo-photolyase with 5 μM $FADH^-$ (○) and (C) 2.5 μM apo-photolyase with 2.5 μM $FADH^-$ (Δ), in strictly anaerobic conditions. The data were fitted by non-linear regression to get the k_1.

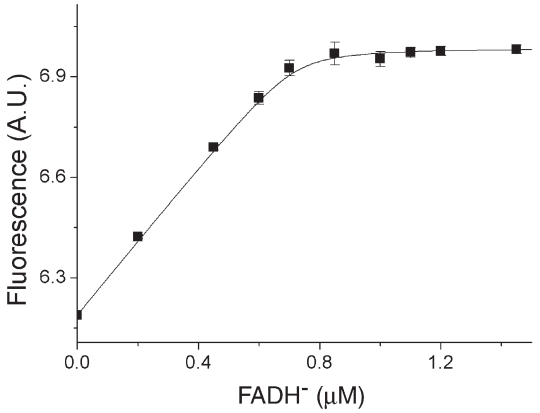

Fig. 7. Determination of the K_D of apo-photolyase to $FADH^-$ by fluorescence titration in anaerobic conditions. The concentration of apo-photolyase was 0.9 μM. The fluorescence emission was measured at 505 nm upon excitation at 360 nm.

$0.19\times10^3\ M^{-1}\ s^{-1}$ and $8.79\pm0.40\times10^3\ M^{-1}\ s^{-1}$ for the concentration of 10 μM, 5 μM and 2.5 μM, respectively. These values are dramatically higher than the value obtained for FAD_{ox} reconstitution and show that apo-photolyase prefers binding with $FADH^-$. k_1 seems to increase with lower concentrations of E and $FADH^-$. One possible explanation for this phenomenon is an oligomer–monomer equilibrium of the apo-photolyase where the monomer binds faster to $FADH^-$.

3.6. Fluorescence titration of apo-photolyase with $FADH^-$

To determine the K_D value of E-$FADH^-$, we titrated 0.9 μM apo-photolyase with $FADH^-$ and monitored the fluorescence change at 505 nm. The fluorescence increased linearly reaching a transition after adding about a stoichiometric amount of $FADH^-$. Further addition of $FADH^-$ did not significantly raise the fluorescence intensity (Fig. 7). From the data, we got the K_D of E-$FADH^-$ to be about 8.3 ± 5.3 nM. Given the relatively high experimental error, this value must be taken with care. Nevertheless, the data do allow to conclude that the estimated upper limit of the K_D for E-$FADH^-$ is about 20 nM. This shows that apo-photolyase tightly interacts with $FADH^-$.

4. Discussion

Photolyase is a flavoprotein that plays an important role in UV-induced DNA lesions repair. In physiological conditions, photolyase contains fully reduced FAD, which is the only active form. Because oxidized FAD is dominant *in vivo*, it was proposed that apo-photolyase binds FAD_{ox} first to form E-FAD_{ox} [1]. However, the actual mechanism of E-FAD_{ox} reduction *in vivo* has not been clearly revealed. Photoreduction has been regarded as a possible reduction route. However, reduced FAD is also associated with photolyase in cells grown in the dark or with a nonphotoreducible mutant of photolyase [1].

To address the enzyme–flavin interaction, we developed a new method to prepare apo-photolyase. For this, freshly prepared photolyase containing neutral FAD radical was treated with 0.5 M imidazole pH 10. Since the free flavin radical has a pK_a value of 8.3 [29], it is conceivable that during the imidazole treatment, anionic flavin radical may generate which more weakly binds to apo-photolyase. Furthermore, the alkaline conditions may weaken the protein–flavin interactions and the heterocyclic imidazole molecule may contribute to that by competing for the flavin binding site.

Apo-photolyase binds $FADH^-$ about two orders of magnitude faster than FAD_{ox} ($\sim3000\ M^{-1}\ s^{-1}$ vs. $34\ M^{-1}\ s^{-1}$). Furthermore, apo-photolyase binds FAD_{ox} relatively weakly (K_D about 2.3 μM) but tightly interacts with $FADH^-$ ($K_D<20$ nM). The K_D of E-FAD_{ox} is slightly higher than that estimated from absorption difference spectroscopy [16]. The strength of interaction between apo-photolyase and FAD is similar to some other flavoproteins that contain reduced flavin cofactors in their active forms, such as luciferase ($K_D\sim120$ μM of FMN_{ox} and ~800 nM of FMN_{red}) [30,31] and chorismate synthase ($K_D\sim30$ μM of FMN_{ox} and ~18 nM of FMN_{red}) [32]. Three mechanisms for how these proteins acquire reduced flavin have been proposed: (a) free diffusion of reduced flavin to an acceptor, (b) reduction of acceptor-bound oxidized flavin by an external reductant including free or bound reduced flavin, and (c) direct channeling of reduced flavin from a donor to an acceptor [33]. Luciferase acquires reduced flavin by direct transfer mechanism [34] and free diffusion is also suggested to occur with some two-component flavin-dependent monooxygenase systems [35,36]. Since apo-photolyase associates with $FADH^-$ much faster than with FAD_{ox}, even if there is little free reduced FAD *in vivo*, apo-photolyase might acquire sufficient $FADH^-$. Our results indicate that *in vivo*, any FAD_{ox} bound will be competitively substituted by $FADH^-$. Because *E. coli* contains several flavin reductases [37,38], the reduced flavin might be provided enzymatically, either through free diffusion or direct channeling. However, it cannot be excluded that photoreduction presents an auxiliary route to ensure sufficient amounts of reduced flavin. The mechanism of flavin transfer to photolyase may be discovered in future studies.

It has been well established that photolyase undergoes no net redox change in catalysis [1,39]. Interestingly, the activity of E-$FADH^-$ in reconstituted photolyase was more than twice that of E-FAD_{ox}. Because substrate was in excess in the activity assays, the enzyme takes several turns in catalysis and E-$FADH^\bullet$ and E-FAD_{ox} must regain their activity after being photoreduced. This suggests that E-FAD_{ox} should have comparable activity with E-$FADH^-$ after a single turnover. The fact that this is not observed supports our finding that the apoenzyme has a much lower binding affinity for FAD_{ox} than $FADH^-$.

During this work we found that the MTHF cofactor of DNA-photolyase can be removed from the protein by washing the immobilized enzyme with deionized water. The polyglutamates tail of the MTHF cofactor has been proposed to increase the binding affinity for the photolyase [1]. However, the enzyme can also bind stoichiometric amounts of the monoglutamate form of MTHF [1,3]. The crystal structure of *E. coli* photolyase shows that the pterin ring of MTHF binds in a shallow protein groove formed by residues 44, 108, 109, 292 and 375, and that the single glutamate of the monoglutamate form of MTHF

forms a salt bridge with Lys293 [3]. Studies from site-directed mutants revealed that Glu109 is critical for MTHF binding [13,14]. Our results show that the binding affinity of MTHF decreases in low ionic conditions, suggesting that hydrophobic interaction is the major force of MTHF binding. This also might explain why the hydrophobic interaction chromatography procedure of apo-photolyase preparation [15] is not suitable for the specific removal of one of the cofactors.

Acknowledgements

We thank Dr. Robert J. Stanley, Dr. Shuqin Yu, Dr. Qinhua Song for discussion and/or experimental help.

The research was supported by the National Natural Science Foundation of China (20273066, 30470444).

References

[1] A. Sancar, Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors, Chem. Rev. 103 (2003) 2203–2237.
[2] M.S. Jorns, G.B. Sancar, A. Sancar, Identification of a neutral flavin radical and characterization of a second chromophore in *Escherichia coli* DNA photolyase, Biochemistry 23 (1984) 2673–2679.
[3] H.W. Park, S.T. Kim, A. Sancar, J. Delsenhofer, Crystal structure of DNA photolyase from *Escherichia coli*, Science 268 (1995) 1872–1886.
[4] M.S. Jorns, B. Wang, S.P. Jordan, DNA repair catalyzed by *Escherichia coli* DNA photolyase contain only reduced flavin: elimination of enzyme's second chromophore by reduction with sodium borohydride, Biochemistry 26 (1987) 6810–6816.
[5] G. Payne, P.F. Heelis, B.R. Rohrs, A. Sancar, The active form of *Escherichia coli* DNA photolyase contains a fully reduced flavin and not a flavin radical, both in vivo and in vitro, Biochemistry 26 (1987) 7121–7126.
[6] G.B. Sancar, F.W. Smith, R. Reld, G. Payne, M. Levy, A. Sancar, Action mechanism of *Escherichia coli* DNA photolyase I. Formation of the enzyme–substrate complex, J. Biol. Chem. 262 (1987) 478–485.
[7] M.S. Jorns, E.T. Baldwin, G.B. Sancar, A. Sancar, Action mechanism of *Escherichia coli* DNA photolyase II. Role of the chromophores in catalysis, J. Biol. Chem. 262 (1987) 486–491.
[8] G.B. Sancar, M.S. Jorns, G. Payne, D.J. Fluke, C.S. Rupert, A. Sancar, Action mechanism of *Escherichia coli* DNA photolyase III. Photolysis of the enzyme–substrate complex and the absolute action spectrum, J. Biol. Chem. 262 (1987) 486–491.
[9] P.F. Heelis, A. Sancar, Photochemical properties of *Escherichia coli* DNA photolyase: A flash photolysis study, Biochemistry 25 (1986) 8163–8166.
[10] J.L. Johnson, S. Hamm-Alvarez, G. Payne, G.B. Sancar, K.V. Rajagopalan, A. Sancar, Identification of the second chromophore of *Escherichia coli* and yeast DNA photolyases as 5,10-methenyltetrahydrofolate, Proc. Nati. Acad. Sci. U. S. A. 85 (1988) 2046–2050.
[11] S. Hamm-Alvarez, A. Sancar, K.V. Rajagopalan, Role of enzyme-bound 5,10-methenyltetrahydropteroylpolyglutamate in catalysis by *Escherichia coli* DNA photolyase, J. Biol. Chem. 264 (1989) 9649–9656.
[12] S. Kim, P.F. Heelis, T. Okamura, Y. Hirata, N. Mata, A. Sancar, Determination of rates and yields of interchromophore (folate–flavin) energy transfer and intermolecular (flavin–DNA) electron transfer in *Escherichia coli* photolyase by time-resolved fluorescence and absorption spectroscopy, Biochemistry 30 (1991) 11262–11270.
[13] A.A. Henry, R. Jimenez, D. Hanway, F.E. Romesberg, Preliminary characterization of light harvesting in *E. coli* DNA photolyase, ChemBioChem 5 (2004) 1088–1094.
[14] E. Schleicher, B. Heßling, V. Illarionova1, A. Bacher1, S. Weber, G. Richter, K. Gerwert, Light-induced reactions of *Escherichia coli* DNA photolyase monitored by Fourier transform infrared spectroscopy, FEBS. J. 272 (2005) 1855–1866.
[15] M.S. Jorns, B. Wang, S.P. Jordan, L.P. Chanderkar, Chromophore function and interaction in *Escherichia coli* DNA photolyase: reconstitution of the apoenzyme with pterin and/or flavin derivatives, Biochemistry 29 (1990) 552–561.
[16] G. Payne, M. Wills, C. Walsh, A. Sancar, Reconstitution of *Escherichia coli* photolyase with flavins and flavin analogues, Biochemistry 29 (1990) 5706–5711.
[17] M.H. Hefti, J. Vervoort, W.J.H. van Berkel, Deflavination and reconstitution of flavoproteins: tackling fold and function, Eur. J. Biochem. 270 (2003) 4227–4242.
[18] W.J.H. van Berkel, W.A.M. van den Berg, F. Müller, Large-scale preparation and reconstitution of apo-flavoproteins with special reference to butyryl-CoA dehydrogenase from *Megasphaera elsdenii*: hydrophobic-interaction chromatography, Eur. J. Biochem. 178 (1988) 197–208.
[19] M.H. Hefti, F.J. Milder, S. Boeren, J. Vervoort, W.J.H. Van Berkel, A His-tag based immobilization method for the preparation and reconstitution of apoflavoproteins, Biochim. Biophys. Acta 1619 (2003) 139–143.
[20] P.F. Heelis, G. Payne, A. Sancar, Photochemical properties of *Escherichia coli* DNA photolyase: selective photodecomposition of the second chromophore, Biochemistry 26 (1987) 4634–4640.
[21] W. Mu, D. Zhang, L. Xu, Y.-B. Wang, Z. Luo, Y. Lou, Y.-Z. Wang, Activity assay of his-tagged *E. coli* DNA photolyase by RP-HPLC and SE-HPLC, J. Biochem. Biophys. Methods 63 (2005) 111–124.
[22] M.S. Jorns, G.B. Sancar, A. Sancar, Identification of oligothymidylates as new simple substrates for *Escherichia coli* DNA photolyase and their use in a rapid spectrophotometric enzyme assay, Biochemistry 24 (1985) 1856–1861.
[23] B. Wang, M.S. Jorns, Reconstitution of *Escherichia coli* DNA photolyase with various folate derivatives, Biochemistry 28 (1989) 1148–1152.
[24] A. Bhattacharyya, G. Tollin, M. Davis, D.E. Edmondson, Laser flash photolysis studies of intramolecular electron transfer in milk xanthine oxidase, Biochemistry 22 (1983) 5270–5279.
[25] Y.M. Gindt, E. Vollenbroek, K. Westphal, H. Sackett, A. Sancar, G.T. Babcock, Origin of the transient electron paramagnetic resonance signals in DNA photolyase, Biochemistry 38 (1999) 3857–3866.
[26] A.W. MacFarlane IV, R.J. Stanley, Evidence of powerful substrate electric fields in DNA photolyase: implications for thymidine dimer repair, Biochemistry 40 (2001) 15203–15214.
[27] F. Perrin, Polarization de la lumiere de fluorescence: Vie moyenne de molecules dans l'etat excite, J. Phys. Radium 7 (1926) 390–401.
[28] S. Ghisla, V. Massey, J. Lhoste, S.G. Mayhew, Fluorescence and optical characteristics of reduced flavines and flavoproteins, Biochemistry 13 (1973) 589–597.
[29] W.M. Clark, in: R.E. Creiger (Ed.), Oxidation Reduction Potentials of Organic Systems, Huntington, NY, 1972, p. 129.
[30] E.A. Meighen, J.W. Hastings, Binding site determination from kinetic data. Reduced flavin mononucleotide binding to bacterial luciferase, J. Biol. Chem. 246 (1971) 7666–7674.
[31] T.O. Baldwin, M.Z. Nicoli, J.E. Becvar, J.W. Hastings, Bacterial luciferase. Binding of oxidized flavin mononucleotide, J. Biol. Chem. 250 (1975) 2763–2768.
[32] P. Macheroux, J. Petersen, S. Bornemann, D.J. Lowe, R.N.F. Thorneley, Binding of the oxidized, reduced, and radical flavin species to chorismate synthase. An investigation by spectrophotometry, fluorimetry, and electron paramagnetic resonance and electron nuclear double resonance spectroscopy, Biochemistry 35 (1995) 1643–1652.
[33] S.-C. Tu, Reduced flavin: donor and acceptor enzymes and mechanism of channeling, Antioxid. Redox Signal. 3 (2001) 881–897.
[34] B. Lei, S.-C. Tu, Mechanism of reduced flavin transfer from *Vibrio harveyi* NADPH-FMN oxidoreductase to luciferase, Biochemistry 37 (1998) 14623–14629.
[35] J. Valton, L. Filisetti, M. Fontecave, V. Nivière, A two-component flavin-dependent monooxygenase involved in actinorhodin biosynthesis in *Streptomyces coelicolor*, J. Biol. Chem. 279 (2004) 44362–44369.
[36] U. Kirchner, A.H. Westphal, R. Müller, W.J.H. van Berkel, Phenol hydroxylase from *Bacillus thermoglucosidasius* A7, a two-protein component monooxygenase with a dual role for FAD, J. Biol. Chem. 278 (2003) 47545–47553.

[37] F. Fieschi, V. Nivière, C. Frier, J.-L. Décout, M. Fontecave, The mechanism and substrate specificity of the NADPH: flavin oxidoreductase from *Escherichia coli*, J. Biol. Chem. 270 (1995) 30392–30400.
[38] B. Galán, E. Díaz, M.A. Prieto, J.L. García, Functional analysis of the small component of the 4-hydroxyphenylacetate 3-monooxygenase of *Escherichia coli* W: a prototype of a new flavin:NAD(P)H reductase subfamily, J. Bacteriol. 182 (2000) 627–636.
[39] Y.T. Kao, C. Saxena, L. Wang, A. Sancar, D. Zhong, Direct observation of thymine dimer repair in DNA by photolyase, Proc. Nati. Acad. Sci. U. S. A. 102 (2005) 10788–10792.