THE
EMBO
JOURNAL

Manuscript EMBO-2010-76185

# Crystal structures of an archaeal class II DNA photolyase and its complex with UV-damaged duplex DNA

Stephan Kiontke, Yann Geisselbrecht, Richard Pokorny, Thomas Carell, Alfred Batschauer, Lars-Oliver Essen

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**Review timeline:**

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| Submission date: | 06 October 2010 |
| Editorial Decision: | 22 November 2010 |
| Revision received: | 14 March 2011 |
| Editorial Decision: | 14 April 2011 |
| Revision received: | 14 July 2011 |
| Accepted: | 02 August 2011 |

**Transaction Report:**

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 November 2010

Thank you again for submitting your manuscript for consideration by The EMBO Journal. I need to profoundly apologize for the fact that it has taken us significantly longer than usual to get back to you with the outcome of its evaluation. The main reason for this is that it has been difficult to readily find a sufficient number of reviewers with the right set of expertise, and that the four reports we eventually received (please find them copied below) were not in best agreement and necessitated asking some further input from some referees. The outcome of these deliberations is, I am afraid to say, that we cannot consider the study a good candidate for publication in The EMBO Journal, at least not in its present form. There are a number of more technical concerns and issues of data interpretation in all four reports; however the overriding main concern is that it is not clear whether the structural data on class II photolyase have really offered sufficient new insights and revealed major differences compared to earlier insights from class I photolyase structures to warrant publication in a broad general journal at this stage. One main point of criticism in this respect is, as pointed out by referee 4, the absence of structure-based mutagenesis and functional analyses to test especially the inferred major class differences, or to validate the functionality of the crystallized complex for photorepair and photoreduction (see also referees 1 and 2). In addition, referee 4 raises an important concern regarding the structural model building - which was only partially alleviated by me providing him/her with the further explanations you offered in our initial correspondence (these extra comments are also copied below).

I do realize that the study may become a much stronger candidate for an EMBO J paper if extended to bolster the functional significance of the findings and to substantiate the experimental evidence in their support, and I also do not want to disregard the interest in principle voiced by some of the

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referees here. Thus, should you be willing and able to conclusively address these issues I would be inclined to give you the opportunity to resubmit a revised version of the manuscript. It is however clear that this will likely entail a considerable amount of further work and effort, and would therefore understand if you were to decide to rapidly publish the study without major changes in a more structural journal. I should also make clear that we would only be able to consider the study further if mutational analysis should really be able to corroborate the proposed functional differences and offer significant new insight compared to previous photolyase structural work - something we would have to decide on in further consultations with referees 1 and 4. Should you choose to resubmit a revised manuscript to The EMBO Journal, please be aware that it is our policy to allow only one single round of major revision, and that it is thus essential to diligently answer to all the major and minor points raised at this stage. Also, when preparing a letter of response, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding this decision.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Kiontke et al. have solved the first structure of a class II photolyase in complex with a synthetic cyclobutane photodimer and note an electron transfer pathway that differs from those previous found in class I photolyases. Moreover, the structure, which was determined at very high resolution, shows a peculiar cluster of water molecules in the active site which may indicate differences in the reaction mechanism when compared to other CPD photolyases. Moreover, the DNA/lesion binding mode appears to be different in this photolyase, and a model for the binding .

The results are interesting and the work is well performed. As such the paper merits publication. However, the paper is at times rather difficult to read and there are a few scientific questions that would need to be addressed before publication.

Also, the results merit a more appealing title such as "Remarkable structural differences between class I and class II CPD photolyases".

To start with a rather confusing part of the text, there are several points at which the clarity of the results and discussion could be improved. In the second halve of page 8, the substitution of an asparagine by a glycine in class II PLs is discussed starting with the arginine, which isn't there in the enzyme discussed here. I propose that this be rewritten from the viewpoint of the enzyme (class) the authors discuss in the current paper.

On page 9 the discussion continues with the electron transfer pathway. Although the enzyme described here contains a tryptophan triad also found in photolyases/cryptochromes from other families, the authors state that in this enzyme this triad is not the relevant one, but that another one conserved in class I enzymes is. Can the authors really exclude the possibility that the former triad is ever used, as their text almost explicitly does? Or is this only based on an analysis of residues conserved in class II PLs?
Moreover, the present manuscript describes this triad as novel on several occasions, whereas by the authors' own admission it has already been postulated by Okafuji et al. for another enzyme. It would appear that a claim to novelty then cannot be made, or am I wrong here?

On page 10 the detailed description of lesion binding starts. In the current structure, neither photorepair of the lesion nor photoreduction of the flavin has occurred. Can the authors elaborate on

possible causes for this? I was under the impression that photorepair happens readily even in crystals of pure photolesion without the enzyme. Was a special data collection technique employed? Did the authors try to obtain a photorepaired structure? I'm asking because one might perhaps even ask whether the current structure may be an inactive artifact.

Page 11 continues with a description of how the enzyme may select a conformation of the photolesion that is prone to easy repair. If the authors will allow me to do so I would like to suggest that this part be rewritten a bit as it is somewhat confusing. By clearly stating what kind of conformations the authors expect to be repair-prone on the one hand and which are expected to be more stable on the other, a significant improvement in clarity may be reached.

Page 12 interestingly notes the presence of a cluster of five water molecules close to the lesion. As the structure was determined from a cryo-cooled crystal, one might ask whether the presence of an ordered cluster is not a cryo-artefact, as has been argued to be the case for many such occurrences. The authors should discuss this as a caveat attached to their detailed description of the cluster's structure. Moreover, I am not sure that the presence of water in the active site points to a change in mechanism as seems to be implied; I may be wrong but I think CPD photolesions can be repaired by X-ray photoelectrons even in solution (the authors will correct me if I am incorrect here).

Finally, A. Glas seems to have provided one of the most salient parts of the structure described here, the CPD photolesion built into DNA; maybe the authors wish to consider including him as a coauthor.

As regards the language, there are a few general points:
-the word order would benefit from careful proofreading by a native speaker. Some examples of sentences the authors may want to change are:

Page 8. "The binding site of the catalytic FAD within MmCPDII resembles structurally" --> "The binding site of the catalytic FAD in MmCPDII structurally resembles"

Page 9. "The utilized duplex CPD-DNA" --> "The duplex CPD-DNA that was used"

Page 15. "in eukaryotes" should be moved somewhere else, for instance to the end of the sentence.

Some examples of other things that might be changed to improve readability:

P8.L18. "Although, both" --> "Although both" (remove comma).

P8.L12. How many C-alpha atoms were superimposed? (To be fair, this information was duly included on page 10 for other superpositions.)

P10.L19. "a deviant orientation". The word "deviant" has many negative connotations; I'm not even sure it is a true adjective. I propose that "a deviating orientation" would be far better.

P11.L22 "position-dependent" --> "in a position-dependent manner" or "position-dependently" (that is to say, it is an adverb rather than an adjective).
P14. "with a dimension of" followed by two dimensions. --> "with dimensions of"

Finally, "flash-frozen" on P.18 (third line from the bottom) should be changed into "flash-cooled" as freezing entails the formation of ice crystals, and the space group on P.19 (first line from the top) should be spelled with a capital P in italic font.

I apologize for my late reply.

Referee #2 (Remarks to the Author):

The manuscript describes the structural and biochemical investigation of a class II bacterial photolyase. Studies include the assays of the biochemical activities and the structural elucidation of the enzyme in free and DNA-bound state. The manuscript is very well-written and technically

sound. Although mainly confirmatory in that it corroborates current mechanistic models on photolyase function, the manuscript should appeal EMBO J readership and can be recommended for publication.

Only a few minor issues:
-The authors might like to explain in more detail certain chemical aspects. For instance the spectroscopic features (e.g. "S1-transition") should be explained in more detailed in order to be fully understood by non-experts.
-Figure 4: I am not sure about the orientation of Asn378. In the oxidized flavin N5 is deprotonated and, therefore, it should receive rather than donate an H-bond - i.e. the Asn side chain should be oriented with the NH2 group towards to flavin (this orientation might change upon flavin reduction).
- I do not think that (page 10) the authors can conclude that there is no photoreduction based on flavin planarity. In many enzymes the flavin is planar even in the reduced state. They should better inspect the color of the crystals after data collection.

Referee #3 (Remarks to the Author):

The present manuscript reports the crystal structure of class II photolyase from the methanogenic archaeon Mrthanosarcina mazei (Mm). The structure reveals that the tryptophan-triad chain for redox-cycle exists between 14 - 15 in Mm. This observation is different from that in class I photolyase, in which the chain exists between 16 and 17. Furthermore, the authors found a cluster of five water molecules next to 3'-thymine base of the UV-lesion, which link the lesion to the FAD cofactor in class II photolyase. This observation suggests mechanistic differences to class I photolyase.
The described work is interesting and scientifically sounds. However, there are several points that need to be addressed by the authors:

Major point
1) In Fig.2B, the solid line indicates the spectrum just after adding DTT without any illumination. However, the spectrum is different from that in Fig.2A. A significant level of absorbance at 590 and 640 is observed in Fig.2B, indicating the presence of semiquinoid state of FADH. There might be two possibilities; 1) addition of DTT without any illumination can induce reduction of FAD in class II photolyase or 2) the dark condition used is not complete. The authors should mention this point in the text or figure legend.
2) For this referee the explanation of EMSA assay (Fig.2E) is not clear when combined with the crystal data. The enzyme is monomer without substrate (Fig.3), but when forming complex with CPD-DNA substrate it exists as dimer (two complx per asymmetric unit) as shown in Fig.5. However the authors' explanation of the result of EMSA assay is "complexation with undamaged DNA occurs along with oligomerization of the enzyme along the deoxyphosphate-backbone". Similar non-specific complexation with DNA should occur to damage-containing DNA. If so, why oligomerization cannot be observed in crystal structure (Fig.5)? Result of EMSA assay can be explained by binding of monomer to several sites? Explanation of EMSA assay and crystal structure should be more clear.

EMSA assay indicate specific binding to substrate occurred between 100-200nM (Fig.2D, E) of the enzyme. Why the authors used higher enzyme concentration for repair assay (4.6 M, Fig.2C),

3) Fig.6A need to be added several explanations and correction(?).
In Fig.6A, H2O molecule should be numbered as shown in Fig.6B.
In Fig.6A, explanation of the "black solid lines" and "black dashed lines" should be added in figure legend. (maybe "pai-stacking interaction" and "salt bridge"?).
The "black solid lines" between 3'-thymine and M379 is correct?

Minor point
Ciation of journal in reference is not uniform.
"Proc Natl Acad Sci USA" and "Proceedings of the National-----------"
"Nucleic Acids Res" and "Nucleic Acids Research"

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Referee #4 (Remarks to the Author):

This manuscript describes the crystal structures of a class II DNA photolyase in the free form and in complex with DNA containing a cyclobutane-pyrimidine dimer (CPD). The authors detail the similarities and differences between this class II enzyme from Methanosarcina mazei and those of three other evolutionary clades, namely class I CPD photolyases, (6-4) photolyases, DASH cryptochromes. Several structural differences in active site residues and a lack of C-terminal helices are noted, although the functional consequences of such changes are not clear since no mutational analysis is provided. The data is generally well described in the manuscript, although it is not clear to me that the new discoveries presented here significantly advance our understanding of photolyase above what was previously known. Based on the major concerns outlined below, I cannot recommend publication of this work as it stands.

Major issues:

1. There are no mutational data presented to substantiate the structure-based hypotheses regarding the functional differences between Class I and II photolyases. This work is entirely a structural study, and it can be argued that mutational analysis guided from the sequence alignments would be much more informative when considering how all classes of photolyases function. Of course, the structures revealed several details that could not have been predicted from the primary structure (e.g., N403). Mutating these residues (and ideally the complementary residue on the Class I enzyme) would greatly increase the significance of this work. It would be especially informative to test the hypothesis that the W388-W360-W381 triad is indeed the electron transfer route. Additionally, both M379 and E301 should be tested for their effects on activity, and a G375N mutant would be interesting to test given that it is one of the few differernces between class II and class I photolyases.

2. More information is needed on the molecular replacement search model (the M. barkeri enzyme) used to determine the apo structure. Despite the fact that the structure of this enzyme is basically correct, the statistics for this 1.5 Angstrom structure suggest that there are errors in the model. I would expect much lower R-factors at this resolution, especially given the low B-factors. Because the search model was listed as "to be published" (p. 7) and "unpublished" (p. 18), I cannot decipher the root cause of the high R-factors. What was the rationale for using this structure, and why was it not presented here? At the very least, the authors need to present the crystal structure of the M. barkeri enzyme--both the phasing and refinement statistics and electron density sufficient to convince the reader that this was a good choice of MR model. Perhaps a comparison of the two structures may also provide additional insight into the Class II enzymes. It is also essential for the authors to show electron density for the M. mazei apo enzyme in addition to the statistics in Table I, as was done for the DNA complex structure. Visual inspection of the model fit to the data is the best gauge of the quality of this model.

Minor issues:

1. It would help those readers unfamiliar with this field to very briefly define the importance of the antenna chromophore, especially given its prominence in the discussion. This could be easily done in the 3rd paragraph of the Introduction.

2. Fig 1B is confusing with little information in the legend. In addition to a more descriptive legend, the authors should use different labels for the various constructs (as opposed to "D2QWB2", etc., and the top construct needs to be defined.

3. Fig 2C: a descriptive legend needed as to what is being measured, and the y-axis should be explicitly defined (265 nm).

4. Fig SI1: the residues described in the text should be marked on the alignment.

5. p.8, bottom paragraph: should N379 be N378?

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[referee 4 in response to author explanation of structure model building:]

"Initially, we obtained crystals of the M. barkeri photolyase (MbPHL) in three different, but only uncomplexed crystal forms without CPD-DNA. The MbPHL structure could be solved at 2.1 Angstrom resolution from the trigonal crystal form (spg. P31, a=b=119.4 Angstrom; 3 molecules per a.s.u.) by border-line molecular replacement using the Thermus thermophilus crystal structure as search model for PHASER and ARP/WARP to get interpretable electron density. During refinement (final R-work/R-free 23.8/27.7 %) we obtained superior crystals of the MmPHL diffracting to 1.5 Angstrom as well as crystals of the desired complexes with duplex CPD-DNA."

So, the authors used a sub-standard model as the basis for solving the higher resoution structure. This is highly unconventional, and strenthens my previous argument. The correct procedure in these types of cases would be to obtain a de novo solution using SAD, MAD, or MIR phasing from heavy-atom derivatives. This would not be an issue if the refinement statistics were what they should be for a 1.5 Angstrom structure. I am convinced that there are errors in this model that have been propogated from the faulty MbPHL search model.

"The MmPHL structures then solved by MR are the scope of the Manuscript sent to you, because we found that the inclusion of the MbPHL structure in this manuscript could be confusing and not helpful per se for the understanding of the function of class II photolyases by the general reader. Our message that our findings on the M. mazei class II photolyase are representative not only for prokaryotic photolyases but the metazoans' as well would be hence diluted."

OK-I can understand this logic for not including the structure in the analysis of photolyases, and especially should not be included if it is not a quality model (as the R-factors given above would suggest). However, I still think that the data collection, MR (very important), and refinement statistics for this model be presented in the Supplemenary Information, as it was used to generate the model presented.

1st Revision - authors' response 14 March 2011

Almost all referees' comments were very helpful for improving the manuscript. Especially referee 1 contributed some questions concerning the clarity of the manuscript that may be of utter importance for the general reader. Besides some additional experimental data on the activity of photolyase crystals (Fig. S2E, S2F), we also followed the suggestion of referee 4 to pinpoint at least some of our conclusions with mutagenesis data. We focused on the unique electron-transfer pathway of class II photolyases and could demonstrate by the W360F mutant the validity of this specific pathway (Fig. 6C-6E). Although we are in the process to generate and analyze more mutants concerning e. g the specific features of the active site and its supported catalysis we feel that this first time description of a close archaeal homolog to metazoan photolyases in functional and structural terms should not be distracted by aspects interesting only for the specialized reader.

Changes of the manuscript & responses to referees' requests:

Referee #1

*"the results merit a more appealing title":*

We changed the title only slightly to "Crystal structures of an archaeal class II DNA photolyase and its complex with UV-damaged duplex DNA". We think that this title together with the accompanying abstract is more convincing than the kind of very general title suggested by ref. #1.

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"*To start with a rather confusing part of the text, there are several points at which the clarity of the results and discussion could be improved.*"

The referee is right. We changed the abstract, straightened the introduction and put the results/discussion into an improved order. After the chapters on phylogeny, bio-/photochemical analyses of the archaeal photolyase its crystal structure we now went on with the complex with duplex DNA before going to the photoreduction pathway and conclusions. By this, we expect that the general reader who is interested in DNA repair will not be distracted by the photolyase/cryptochrome-specific issue of photoreduction pathways.

*"... this be rewritten from the viewpoint of the enzyme (class) the authors discuss in the current paper".*

The referee is right to ask for a proper focus on class II. We rephrased the corresponding section (page 17).

*"Can the authors really exclude the possibility that the former triad is ever used, as their text almost explicitly does? Or is this only based on an analysis of residues conserved in class II PLs?"*

The referee is right in getting this point better clarified. As stated now in the text (page 18, top) the class I electron transfer pathway is simply not available in class II photolyases according to sequence and structural analysis. As also suggested by ref. #4, we performed mutagenesis studies on the two innermost tryptophanes (W360, W381) of the tryptophane triade. Whereas the variant with the inner tryptophane mutated (W381F) was apparently not properly folded under our expression conditions, the medial W360F variant showed 15fold reduction of activity. We included these data in Fig. 6D and 6E and added corresponding text (page 18, bottom)to the end of the section "FAD-binding site and electron transfer pathways in class II PHL". Finally, we omitted to claim of "novelty" for the tryptophane triade of class II pathways, because this pathway has been suggested before by Okafuji et al.

*"In the current structure, neither photorepair of the lesion nor photoreduction of the flavin has occurred. Can the authors elaborate on possible causes for this? I was under the impression that photorepair happens readily even in crystals of pure photolesion without the enzyme. Was a special data collection technique employed? Did the authors try to obtain a photorepaired structure? I'm asking because one might perhaps even ask whether the current structure may be an inactive artifact."*

The referee is right to ask. We put our analysis of the base tilt angles of CPD lesions into this context, because we observe that in MmCPDII the CPD structure is very similar to the CPD lesions in several other crystal structures. Although we already cited reports in Mees et al. (Science, 2004) that CPD lesions may become repaired by X-rays in frozen solvent matrices, other structures apart than the CPD photolyase from A. nidulans and cryptochrome 3 from A. thaliana did not show the kind of X-ray repaired CPD-lesions (Fig. S3). To exclude that the crystals of the MmCPDII/CPD-DNA complex are photochemically inactive we performed microspectroscopic experiments at the synchrotron (Fig. S2E, S2F) clearly showing that the crystallized MmCPDII is capable to undergo photoreduction as well as X-ray mediated reduction. Here, we did not use a rapid or exposure-limited data recording strategy. Attempts to follow X-ray induced structural changes by the recording of highly overredundant datasets were not yet able to show any X-ray mediated bond breakage within the cyclobutane ring of the CPD lesion. Our attempts to obtain in situ repair by light exposure have been not yet successful, maybe due to the acidic pH at which the crystals can be handled in a stable manner.

"...continues with a description of how the enzyme may select a conformation of the photolesion that is prone to easy repair. If the authors will allow me to do so I would like to suggest that this part be rewritten a bit as it is somewhat confusing. By clearly stating what kind of conformations the authors expect to be repair-prone on the one hand and which are expected to be more stable on the other, a significant improvement in clarity may be reached."
The referee is right. To answer his previous questions we indeed rewrote and sharpened this section

of the manuscript.

*"Page 12 interestingly notes the presence of a cluster of five water molecules close to the lesion. As the structure was determined from a cryo-cooled crystal, one might ask whether the presence of an ordered cluster is not a cryo-artefact, as has been argued to be the case for many such occurrences. The authors should discuss this as a caveat attached to their detailed description of the cluster's structure."*

The referee is partly right. Clearly, water cluster formation along protein surfaces can be induced by freezing and especially in the case of a protein optimized in electron transport reactions this is a matter of concern. However, we observe the water cluster already in the uncomplexed state as noted in the text. As the water cluster is occluded from the bulk water by the bound CPD lesion, it cannot be expected that in the different contexts of the bound and unbound state an almost identical water structure forms.

*"As regards the language, there are a few general points: ..."*

Many thanks to referee 1. We followed the ref. #1 suggestions, implemented the suggested corrections and obtained support by a native speaker.

Referee #2

*"The authors might like to explain in more detail certain chemical aspects. For instance the spectroscopic features (e.g. "S1-transition") should be explained in more detailed in order to be fully understood by non-experts."*

The referee is right. We omitted the quite specific spectroscopic aspect of the S1-transition observable in oxidized flavin spectra, because it doesn't contribute to the understanding of this manuscript.

*"Figure 4: I am not sure about the orientation of Asn378. In the oxidized flavin N5 is deprotonated and, therefore, it should receive rather than donate an H-bond - i.e. the Asn side chain should be oriented with the NH2 group towards to flavin (this orientation might change upon flavin reduction)."*

The referee is right. In all available structures of photolyases and cryptochromes the sidechain of the crucial asparagine points with its carboxamide's oxygen towards the N5 nitrogen of FAD. This orientation as mentioned in the text (page 17) makes sense as the sidechain makes other, secondary hydrogen bonds proton acceptors nearby. Likewise, N403 makes an H-bond to D404 and the FAD's O4 oxygen atom via its carboxamide amine.

*"I do not think that (page 10) the authors can conclude that there is no photoreduction based on flavin planarity. In many enzymes the flavin is planar even in the reduced state. They should better inspect the color of the crystals after data collection."*

The referee is right. Indeed the absence of buckling is not giving any hint about the redox state of flavins. Accordingly, we included our microspectroscopic data on crystals to show that the oxidized species (page 11, second para, Figs. S2E, S2F) has been indeed crystallized, but undergoes X-ray induced reduction of the chromophore as we and others (e. g. Kort et al., 2004) have generally observed for photolyases.

Referee #3

*"In Fig.2B, the solid line indicates the spectrum just after adding DTT without any illumination. However, the spectrum is different from that in Fig.2A. A significant level of absorbance at 590 and 640 is observed in Fig.2B, indicating the presence of semiquinoid state of FADH. There might be two possibilities; 1) addition of DTT without any illumination can induce reduction of FAD in class II photolyase or 2) the dark condition used is not complete. The authors should mention this point in*

*the text or figure legend."*

The referee is right, very gratefully, although in a different way, because we erroneously included the wrong spectrum in Fig. 2B. This figure is now corrected and shows that the addition of DTT in the dark has indeed no effect on the spectrum of MmCPDII.

*"the explanation of EMSA assay (Fig.2E) is not clear when combined with the crystal data. The enzyme is monomer without substrate (Fig.3), but when forming complex with CPD-DNA substrate it exists as dimer (two complx per asymmetric unit) as shown in Fig.5. However the authors' explanation of the result of EMSA assay is "complexation with undamaged DNA occurs along with oligomerization of the enzyme along the deoxyphosphate-backbone". Similar non-specific complexation with DNA should occur to damage-containing DNA. If so, why oligomerization cannot be observed in crystal structure (Fig.5)? Result of EMSA assay can be explained by binding of monomer to several sites? Explanation of EMSA assay and crystal structure should be more clear."*

The referee got apparently confused by our order of presentation. The crystal packing has per se no obvious relationship to the situation in solution. As the aspect of crystal packing is of lower value for the general reader we put the figure into the supplementary material (Fig. S1B) in order not to distract the reader from the characterization of the MmCPDII/CPD-DNA complex.
The referee is right in mentioning unspecific binding in the EMSA for CPD-comprising oligonucleotides. Indeed we observe this phenomenon (Fig. 2E) at high enzyme concentrations. The text has been accordingly changed at the end of the para "UV/VIS-spectra, repair activity and DNA-binding of MmCPDII":
"Accordingly, MmCPDII distinguishes in terms of affinity only marginally between CPD damaged and undamaged duplex DNA with KD,NS/KD,S ratios of ~ 3. The found Hill coefficient of 3.9 indicates that complexation with undamaged DNA will proceed by the binding of several enzyme molecules along the deoxyribophosphate-backbone, if a huge excess of enzyme is present. In contrast, for the specific recognition of a CPD lesion within DNA a single enzyme molecule suffices (Figure 2E), although at high enzyme concentrations larger complexes become likewise apparent."

*"EMSA assay indicate specific binding to substrate occurred between 100-200nM (Fig.2D, E) of the enzyme. Why the authors used higher enzyme concentration for repair assay (Fig.2C)"*

We had to use the high concentrations of MmCPDII (4.6 micromolar) in the spectroscopic photo repair assay due to the high concentrations of substrate needed for producing a significant signal of absorbance change. Lower enzyme concentrations as used in the EMSA (<=1 micromolar) would have prolonged the assay beyond the stability of enzyme and substrate.

*"Fig.6A need to be added several explanations and correction(?)."*
The referee is right. We improved the figure legend according to ref. #3 suggestions.

Citations were corrected as suggested.

Referee #4
*"There are no mutational data presented to substantiate the structure-based hypotheses regarding the functional differences between Class I and II photolyases. This work is entirely a structural study, and it can be argued that mutational analysis guided from the sequence alignments would be much more informative when considering how all classes of photolyases function."*

The referee is only partly right. This work is despite its title not only a structural study of MmCPDII, but after an initial and still only analysis of the distant class II photolyase from M. thermoautotrophicum (Kiener et al., 1989; JBC 264, 13880) also the first report on the photochemical and catalytic properties of an archaeal photolyase that is highly related to the metazoan enzymes. However, the request for the analysis of mutants is very useful and we focussed our work on the class II-specific electron transfer pathway. As outlined above in the response to referee 1, we could show by the W360F mutant that the pathway W388 W360 W381 is indeed used by class II photolyases. Further work will be certainly necessary to elucidate potential bypasses and

the pecularities of flavin chemistry within class II enzymes or their precise mode of CPD-repair.

*"More information is needed on the molecular replacement search model (the M. barkeri enzyme) used to determine the apo structure. Despite the fact that the structure of this enzyme is basically correct, the statistics for this 1.5 Angstrom structure suggest that there are errors in the model. I would expect much lower R-factors at this resolution, especially given the low B-factors." and after response to authors' explanation*
*"So, the authors used a sub-standard model as the basis for solving the higher resoution structure. This is highly unconventional, and strengthens my previous argument. The correct procedure in these types of cases would be to obtain a de novo solution using SAD, MAD, or MIR phasing from heavy-atom derivatives. This would not be an issue if the refinement statistics were what they should be for a 1.5 Angstrom structure. I am convinced that there are errors in this model that have been propogated from the faulty MbPHL search model."*

The referee is only partly right. The reported R-factors for the 1.5 Angstrom structure of MmCPDII are not unusual at all. Currently (14-3-2011), the RCSB protein database has 2055 entries with resolutions of 1.47-1.55 Angstrom. Of these entries, about a half (1107) have a free R-factor (the only sensible value) of 21 % or better, 948 of worse than 21 %. 323 have a free R-factor between 20 and 21 %. In terms of statistics our 1.5 Angstrom structure is quite average. Our quite conservative approach during the refinement of MmCPDII was to avoid bias, which gave the final working R-factor of 18.5 % and the rather small gap of 2.5 % between both kind of R-factors. This indicates that the degree of deviation of the structural model from the "real" structure is rather small. I do not see any sense to push the R-factor by increasing the gap to R-free and introducing additional bias by relaxed modeling. As a further safeguard, we also continued the independent refinement of the M. barkeri photolyase (Suppl. Table I) and compared the results later. There is indeed a lack of structural definition of the linker region, particularly in the MbCPDII, which cannot be overcome, according to my experience, by the recording of experimental phases (SAD etc.) as suggested by referee #4. Intrinsic disorder of larger parts of the structure is quite refractory to modelling and often causes higher R-factors than fully defined structures.

*"Minor issues:*
*1. It would help those readers unfamiliar with this field to very briefly define the importance of the antenna chromophore, especially given its prominence in the discussion. This could be easily done in the 3rd paragraph of the Introduction.*

The referee is right. We added "Diverse classes of antenna chromophores like MTHF, 8-hydroxydeazaflavin, FMN or FAD have been identified in some photolyases/cryptochromes to broaden their activity spectra, whereas many others apparently lack any bound antenna chromophores." to the suggested para.

*2. Fig 1B is confusing with little information in the legend. In addition to a more descriptive legend, the authors should use different labels for the various constructs (as opposed to "D2QWB2", etc., and the top construct needs to be defined.*

The referee is right. We improved the figure legend.

*3. Fig 2C: a descriptive legend needed as to what is being measured, and the y-axis should be explicitly defined (265 nm).*

The referee is right. We corrected as suggested.

*4. Fig SI1: the residues described in the text should be marked on the alignment.*

We followed the advice of the referee to make the fig. SI4 (previously SI1) more usable by splitting it into two subsections A and B, one for the structure-based alignments between the subfamilies (A) and one specific for class II (B). The relevant residues are then highlighted by colouring as explained in the Figure legend.

*5. p.8, bottom paragraph: should N379 be N378?"*

The referee is right. We were misled by the nomenclature for numbering that is defined in the legend text of Fig. S4 as it definitely refers to the UniProt entries. However, the E. coli coordinates' entry 1DNP differs by -1 from this numbering scheme, as the N-terminal start methionine was not considered at the time of its deposition. As it is common sense to stick to the E. coli numbering (and we actually used it in figure 6) we corrected to N378.

2nd Editorial Decision 14 April 2011

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Thank you for submitting your revised manuscript for our editorial consideration. The original referees 1 and 4 have now once more looked into it, as well as at your response letter to their original points of criticism. Both consider the manuscript significantly improved over the original version, especially with regard to many of the textual and technical concerns; we would therefore in principle be prepared to consider the manuscript further for ultimate publication in The EMBO Journal. However, I am afraid the study still falls short with regards to deeper structure-based mutagenesis and subsequent functional analysis, as had been explicitly requested in my original letter; given the relative maturity of the photolyase field, we feel that such further mutagenesis-based query of the implied major structural differences would nevertheless remain a prerequisite for publication in a broad general journal at this point. In this respect, the added information from mutation of the W360 residue is clearly appreciated, but as pointed out by referee 4, none of the other originally proposed residues apart from the 'tryptophan triad' have been scrutinized in this manner.

Given the interest of the topic and the major improvements in the revised paper (in the face of adverse circumstances, which we are certainly aware of), we are willing to grant an additional round of major revisions in this case, in order to allow you to conduct further mutagenesis analyses to complement the structural data, as originally requested. In this respect, referee 4 offers a few additional (alternative) suggestions in his/her report on the revised manuscript (copied below). Should you be able to add informative data on at least a subset of additional mutations with different projected roles (such as N403) to shed light on the differences between class I and class II photolyases, then we could ultimately consider publication in The EMBO Journal. In addition, referee 4 also retains a few other issues which we feel will not demand new data, but nevertheless require adequate and thorough discussion in a re-revised manuscript, especially to accentuate the novel insights into UV repair gleaned from the class II photolyase structure.

I realize that such additional experiments will require considerable further efforts, but we maintain that they would be necessary to advance this work to a compelling high-impact publication suitable for a broad non-specialist journal at this stage, as already explained in my original letter. We would therefore be willing to grant another three months revision period to accommodate this. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

In any case, please do not hesitate to get back to me should you have any further questions or comments regarding this decision or your re-revision.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Kiontke and coworkers' structural characterization at high resolution of the first member of a class II photolyase is of high interest to the field. In particular, the novel active site structure containing a water cluster and its unusual tryptophan triad set this structure apart from other photolyase structures.

Moreover, they authors have have adequately addressed the points I raised earlier. Indeed, the revisions the authors have made render the paper far more readable and attractive. In it's current form, the paper is suitable for publication in EMBO journal.

Referee #4 (Remarks to the Author):

In their revised manuscript, Essen and colleagues describe the crystal structure and biochemical characterization of a Class II photolyase, which bears overall resemblance to the other subclasses of photolyases but contains noteworthy differences at the side-chain level. The authors do a nice job at describing the structural results and have made an attempt at using the structure to explain the mechanism of electron transfer using mutagenesis. These results modestly enhance our understanding of photolyases, and this work may be of specific interest to the photolyase community. The data is of good quality and the interpretations are sound.

It seems that there is potential for this enzyme to enhance our understanding of DNA repair (the unrepaired CPD lesion in the active site is a noteworthy observation). However, I missed a meaningful discussion of the relevance of this structure to DNA repair in the revised manuscript, and the authors did not clarify in the abstract and introduction why results from a Class II enzyme enhance our understanding of these enzymes. I still do not get a sense of the functional differences between evolutionary clades. I found the introduction a bit hard to follow in part due to grammatical and typographical issues, although this is a minor point.

The authors did a minimal job at respond to my previous criticisms regarding the lack of mutagenesis to support their claims. The effect is W360F was a nice addition to the discussion of electron transport through the enzyme. But given the number of interesting differences between this structure and other photolyases, I was surprised to find that this was the only residue mutated (a second residue was mutated but caused the protein to unfold). Why were none of the tyrosine residues (Y345, Y442, Y445) implicated by the structure in "electron transfer to the catalytic cofactor by acting as terminal points of this pathway" (p 19) mutated? Similarly, N403 was postulated to play the FAD stabilizing role as Class I N378, which is located on a different helix, yet was not tested. Finally, would disruption of the D428/R441 salt bridge by mutation of D428 increase the activity by freeing R441 to attain its DNA binding position? Because at this point the structural biology of photolyase field is rather mature, demonstrating the functional significance of the differences observed between Class II and the other photolyases is, in my mind, essential for this study to be of interest to the DNA repair community.

Additional points:

I'm having trouble reconciling the EMSA gels in Fig 2E and the quantitation in Fig 2D. Why in 2D does the percentage of bound CPD oligo go to 100% when in the gel the specific complex never constitutes 100% of the bound form? Half way through the titration a 50/50 mixture of specific/unspecific is observed, and the specific complex is then subsumed by formation of unspecific complex. Mis-quantitation would obviously affect the reported Kd values, although perhaps not to an extent to change the authors conclusions. Also, if the non-specific band represents more than one protein bound to the DNA, why is a single protein bound not observed as well, which should be statistically possible unless there is a sequence preference of the enzyme. These seem like interesting results that merit a comment in the manuscript.

I am still confused by the description of antenna chromophores in the introduction and results. Why is the lack of an antenna chromophore (p7-8) so interesting? How often do these enzymes typically contain both FAD and another chromophore, and what is its significance?

2nd Revision - authors' response 14 July 2011

Below you will find our detailed comments on the changes and suggestions made by the referees, primarily referee 4. We followed the very helpful suggestions of referee 4 to pinpoint our conclusions with mutagenesis data. In this context, we focused on the unique electron-transfer pathway of class II photolyases and could demonstrate by mutagenesis studies that the pathway is actually not a triad and is more like a dyad with distinct branches.

Changes of the manuscript & responses to referees' requests:

In general

To present consistent results of the photoreduction assay for the photolyase wild type and mutants we decided to repeat the experiments with freshly prepared wild type enzyme. As a result, we got a shortened half time (t1/2) of the wild type photolyase from t1/2=112 s ± 1 s to t1/2=79.0 s ± 0.5 s. The explanation for this slight difference is that the first experiments were performed with enzyme preparations which were a few weeks old.

To improve the general structure of the manuscript we split the former Figure 6 into two separate figures (now Figure 6 and Figure 7). Due to length considerations some parts of the Materials & Methods section are now presented in the Supplemental Information section.

Referee #4

*"I found the introduction a bit hard to follow in part due to grammatical and typographical issues, although this is a minor point."*

We checked the manuscript for grammatical and typographical issues and made especially the introduction a bit more focused in the first half.

*"The authors did a minimal job at respond to my previous criticisms regarding the lack of mutagenesis to support their claims. The effect is W360F was a nice addition to the discussion of electron transport through the enzyme. But given the number of interesting differences between this structure and other photolyases, I was surprised to find that this was the only residue mutated (a second residue was mutated but caused the protein to unfold)."*

Indeed, the referee was right, at least at that time. We have now eight mutants prepared and analyzed, of which six were very helpful for strengthening our points obtained from the structures. Unfortunately, progress is a bit impeded by the high GC-content (mutagenesis) and the stability of many mutants.

*"Why were none of the tyrosine residues (Y345, Y442, Y445) implicated by the structure in "electron transfer to the catalytic cofactor by acting as terminal points of this pathway" (p 19) mutated?"*

The referee is right to ask for mutants of these possible involved tyrosines. We mutated tyrosine Y345 as well as tyrosine Y380 which may act as an electron transfer bypass for the W360F mutant for a phenylalanine in each case. Furthermore, we were able to produce soluble protein of the innermost W381F and the surface exposed W388F mutant of the suggested tryptophane triad. Our further studies showed that the electron transfer pathway is not necessarily a triad and the knock out of Y380, Y345 or W388 has no or just a negligible effect. The innermost (W381) and the median (W360) tryptophane are the crucial residues for photoreduction and the electron transfer pathway is more like a dyad with distinct branches.

*"Similarly, N403 was postulated to play the FAD stabilizing role as Class I N378, which is located on a different helix, yet was not tested."*

The referee is right to ask for mutations of that really interesting amino acid. We mutated asparagine

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N403 for a nonpolar alanine (N403A), a hydrophobic leucine (N403L) and an acidic aspartate (N403D), respectively. The results are now included in the current version of the manuscript.

At this point we would like to thank referee #4 for his very helpful comments and suggestions. In our opinion, these additional mutation studies helped considerably to improve our manuscript.

*"Finally, would disruption of the D428/R441 salt bridge by mutation of D428 increase the activity by freeing R441 to attain its DNA binding position?"*

Of course, the referee is right to ask for mutational studies regarding DNA binding and repair activity. But, in our additional studies we focused our interest on photoreduction and related aspects. Experiments to determine DNA binding and repair activity need much more efforts to obtain proper results for publication. Here, we would need to compare repair activities of several mutants against different DNA substrates, single-stranded as well as loop-comprising duplex strands in terms of binding and repair activity. Although technically feasible and done with AtCry3 wild type (Pokorny et al., 2008) these studies are too time intensive and would dilute the scope of the manuscript.

*"I'm having trouble reconciling the EMSA gels in Fig 2E and the quantitation in Fig 2D. Why in 2D does the percentage of bound CPD oligo go to 100% when in the gel the specific complex never constitutes 100% of the bound form? Half way through the titration a 50/50 mixture of specific/unspecific is observed, and the specific complex is then subsumed by formation of unspecific complex. Mis-quantitation would obviously affect the reported Kd values, although perhaps not to an extent to change the authors conclusions. Also, if the non-specific band represents more than one protein bound to the DNA, why is a single protein bound not observed as well, which should be statistically possible unless there is a sequence preference of the enzyme. These seem like interesting results that merit a comment in the manuscript."*

The referee is right. In this context, "100 %" is the amount of total bound oligonucleotide. To avoid misunderstandings, we changed the labeling of the y-axis to "Total bound DNA-oligomer / %" in Figure 2D. We would like to thank referee #4 to encourage us to think about the quantitation of our results. Furthermore, we evaluated the data shown in Figure 2D not only by the simple Hill function, but also by a modified Hill equation representing a very simple model for specific and unspecific binding: . This part is also discussed in the current version of the manuscript.

*"I am still confused by the description of antenna chromophores in the introduction and results. Why is the lack of an antenna chromophore (p7-8) so interesting? How often do these enzymes typically contain both FAD and another chromophore, and what is its significance?"*

The referee is right to ask for the significance of the description of antenna chromophores in the introduction. In our opinion it is important to mention that photolyases can harbor an additional cofactor to broaden their activity spectra. UV/Vis spectra of the same photolyase with or without an additional cofactor can exhibit obvious differences. The analysis of the nature of the cognate antenna chromophore of MmCPDII is part of another ongoing study of us.

Acceptance letter 29 July 2011

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Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and I am happy to inform you that we can now consider it suitable for publication in The EMBO Journal.

Yours sincerely,
Editor
The EMBO Journal

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