Thermodynamics of the Photoenzymic Repair Mechanism Studied by Density Functional Theory

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Abstract: The thermodynamics of the different steps in the photoenzymic fragmentation of a thymine dimer is investigated using density functional theory (DFT) methods, including time-dependent (TD) DFT for calculating electronic transition energies, employing a model system consisting of different thymine derivatives and enzymatic cofactors ($FADH^-$, 8-HDF, and MTHF). It is found that the crucial electron-transfer steps, as well as the overall reaction, are exothermic and that the splitting of the C6−C6′ bond in a thymine dimer radical anion is slightly exothermic (2.4 kcal mol^{-1}) with a 2.3 kcal mol^{-1} energy barrier. The reaction energies assigned to the different steps are generally in good agreement with the corresponding energies from previous estimates that have constituted the foundation of the proposed reaction mechanism. On the basis of this comparison, the results support the proposed model. Moreover, the excellent agreement between theoretical excitation energies and experimental data shows that TDDFT can be successfully applied to large organic molecules.

Introduction

Exposure of DNA to ultraviolet (UV) radiation has a mutagenic effect on cellular systems. A major type of UV-induced damage is the formation of cyclobutane pyrimidine dimers[1] (Pyr<>Pyr) from adjacent pyrimidine bases in DNA exposed to far-UV (200−300 nm) radiation (Figure 1). The Pyr<>Pyr are harmful to cells since they inhibit the enzymes carrying out DNA replication and transcription. Photoreactivation by concurrent or subsequent exposure to near-UV and visible light (300−500 nm) is one of the repair processes that cells use to protect themselves against these effects. In the photoreactivation process, the Pyr<>Pyr are transformed into individual pyrimidine bases in a reaction catalyzed by DNA photolyase. The commonly accepted model[2] proposes that the dimer splitting is a consequence of a single electron transfer from the enzyme to the dimer.

DNA photolyases are monomeric proteins[3,4] that are widespread in nature. They have been found in different bacteria as well as in fungi, higher plants, and vertebrates. The enzymes consist of 454 to 614 amino acids[5] and have two noncovalently attached prosthetic groups[6] (Figure 2). One of these is always the deprotonated form of the catalytic cofactor $FADH_2$ (1,5-dihydroflavin adenine dinucleotide[7−9]), and the other, which is a light-harvesting cofactor, can be either a folate[10] (methenyltetrahydrofolate, MTHF) or a deazoflavin[11] (8-hydroxy-5-deazariboflavin, 8-HDF). DNA photolyases have accordingly been divided into two broad groups:[12,13] the folate class with enzymes that bind $FADH^-$ and MTHF and are catalytically active when exposed to radiation of 360−390 nm; the deazoflavin class with enzymes that bind $FADH^-$ and 8-HDF, and have an action spectrum of 430−460 nm. The three-dimensional crystallographic structures of enzymes belonging to these groups have been determined at a resolution of 2.3 (MTHF-DNA photolyase from *Escherichia coli*)[14] and 1.8 Å (8-HDF-DNA photolyase from *Anacystis nidulans*[15]), respectively. Neither DNA-recognizing nor electron-transferring enzymes are unusual

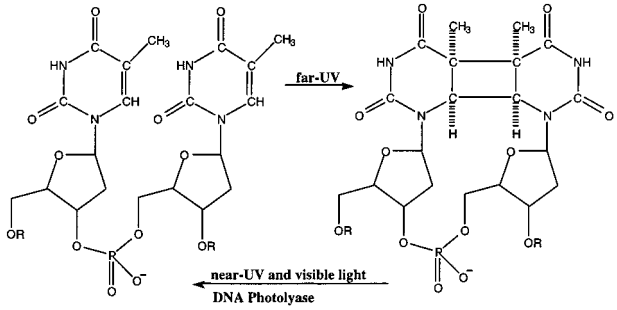

Figure 1. UV-induced thymine dimer formation between two adjacent thymine residues in the same strand of a DNA molecule. The cycloreversion reaction is catalyzed by DNA photolyase, which uses near-UV and visible light.

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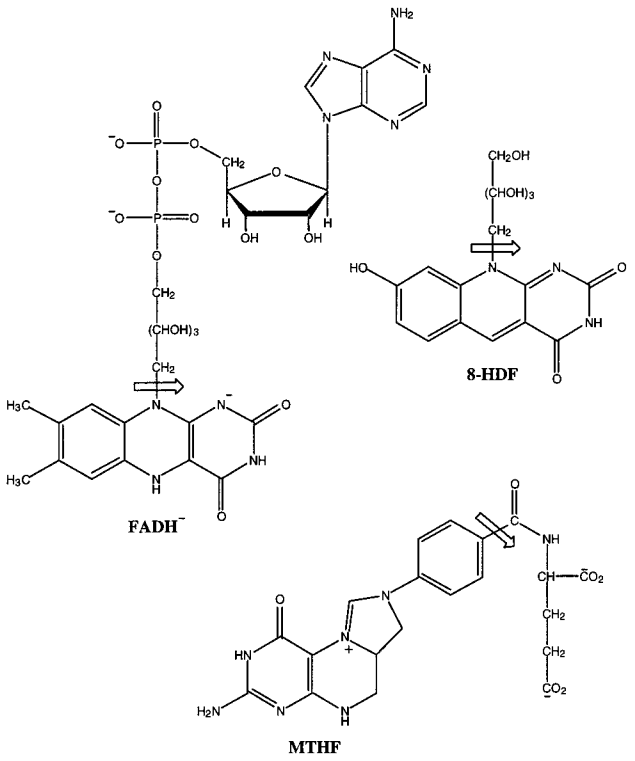

Figure 2. The structures of the catalytic cofactor $FADH^-$ and the light-harvesting cofactors MTHF and 8-HDF. In this study, the side chains of the cofactors are replaced by methyl groups as indicated by the arrows.

in biological systems;[16] the main feature that distinguishes DNA-photolyase from other enzymes is that the catalysis is initiated by light.

The cycloreversion reaction that splits a thymine dimer in DNA serves as an illustrative example of the Woodward−Hoffmann rules of orbital symmetry.[17] Accordingly, a neutral thymine dimer cannot revert to two thymines in a nonphotochemical process. The photochemical cycloreversion reaction is, on the hand, symmetry allowed. This reaction will, however, not take place since thymine dimers do not efficiently absorb light as they lack the conjugated π system of the original thymines. Instead, the dimer is reverted in an anionic reaction process catalyzed by DNA photolyase. The proposed reaction mechanism can be summarized as follows[2,16,18] (Figure 3).

The enzyme recognizes and binds to a thymine dimer in DNA independently of light. The light-harvesting cofactor (MTHF or 8-HDF) absorbs a photon, and the excitation energy is transferred to the catalytic cofactor ($FADH^-$), which in turn is excited (both excitations are singlet $\pi-\pi^*$ transitions from the HOMO to the LUMO). The enzyme then transfers an electron from the excited $FADH^-$ to the dimer whereby the C5−C5′ σ bond of the dimer splits (Figure 4), and a thymine dimer radical anion is formed. The C6−C6′ σ bond of the dimer radical anion subsequently breaks, forming a thymine and a thymine radical anion. The thymine radical anion donates an electron back to the FADH-radical to regenerate $FADH^-$. Finally, the enzyme dissociates from the now intact DNA. The dimer splitting is, apparently, a consequence of an electron transfer from the

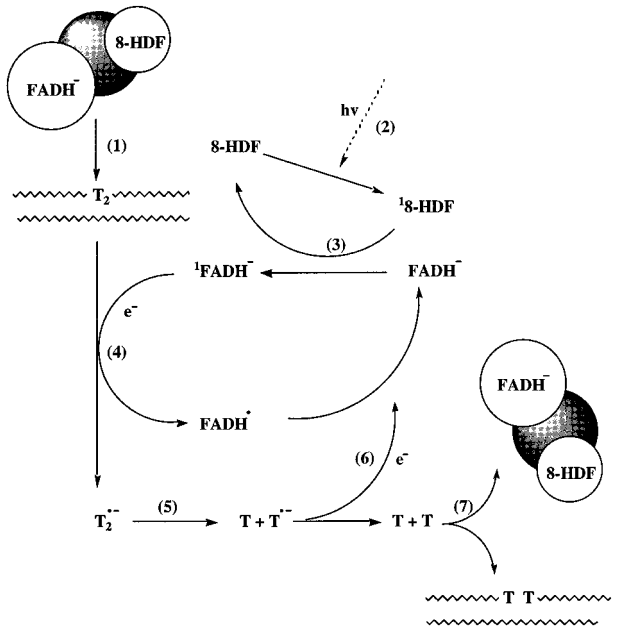

Figure 3. Proposed reaction mechanism of a DNA photolyase belonging to the deazoflavin class:[2,16,18] (1) binding of the enzyme to the thymine dimer; (2) photon absorption by 8-HDF; (3) energy transfer to $FADH^-$; (4) electron transfer to the thymine dimer; (5) fragmentation of the thymine dimer radical anion; (6) back electron transfer to the FADH-radical and regeneration of a catalytically active $FADH^-$; and (7) dissociation of the enzyme from the DNA.

enzyme to the dimer, and is not a redox reaction since there is no gain or loss of electrons during the process. The net result of the photoreactivation process is therefore a transformation of a thymine dimer into two thymine residues. The regeneration of $FADH^-$ also reassures that the enzyme will be ready to perform another catalytic cycle as soon as it has dissociated from the DNA.

The above model is mainly founded on thermodynamic arguments[2,16,18−21] and isotope effect studies.[22,23] However, the uncertainties in the free energy changes assigned to the electron-transfer steps and the splitting of the dimer radical anion could be significant.[16] It is therefore of interest to investigate the thermodynamics of the photoreactivation process theoretically by means of computational chemistry. In this study, the thermodynamics is calculated using density functional theory (DFT) methods, and is compared with the corresponding energies according to Heelis et al.[16,18] Agreement between the different sets of data would justify the model. We also determine the energy barrier for the breakage of the C6−C6′ bond in the dimer radical anion. This is probably a crucial step, and a low barrier would support the proposed anionic reaction mechanism.

The electronic transition energies are calculated by using a recent implementation of time-dependent (TD) DFT[24] available in the Gaussian 98 program.[25] A comparison with experimental energies would indicate whether this method gives satisfactory results when applied to large organic molecules.

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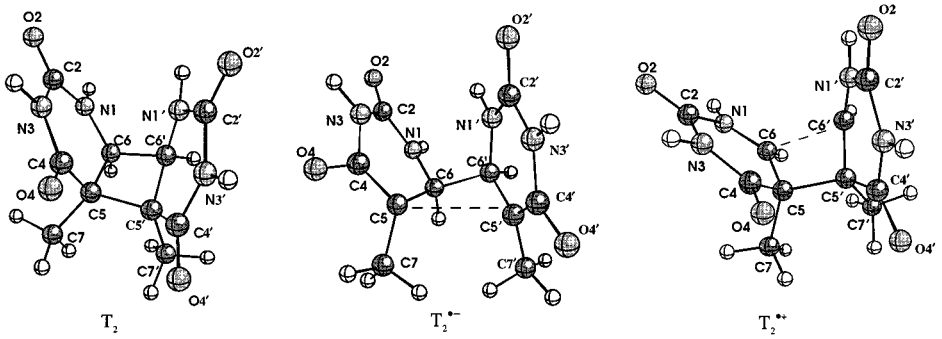

Figure 4. Geometry optimized structures of thymine dimer and its radicals. Numerical values are listed in Table 1.

The reaction mechanism outlined above is somewhat idealized since we have assumed that we restore the thymine residues in their original forms. However, the thymine radical anion, and probably also the thymine dimer radical anion, is easily protonated.[26,27] It is therefore possible that the overall reaction may regenerate modified thymines. Furthermore, the protonation of the dimer radical anion may influence both the kinetics and the thermodynamics of the cycloreversion reaction. On the basis of B3LYP/6-31G(d) and AM1 calculations, Rösch et al. have showed that the activation energy for the initial splitting of the C5−C5′ bond is increased by roughly 10 kcal mol^{-1} (from 0 to 10 kcal mol^{-1}) when a uracil dimer radical anion is protonated.[28]

Methods

All calculations reported in the present work were carried out with the Gaussian 94 program[29] except where otherwise noted. The calculations concerning the thymine dimer focused on the *cis-syn* stereoisomer, which is the predominant form in UV-irradiated DNA.[30]

Simplified models of the prosthetic groups MTHF, 8-HDF, and FADH$^-$ were used throughout the study. The models, shown in Figure 2, seem reasonable from a photochemical/biochemical point of view as the light is absorbed by the conjugated π systems of MTHF and 8-HDF, and the electron is donated from the isoalloxazine ring of FADH$^-$.

Theoretical determination of the gas-phase electron affinities of nucleic acid bases is a difficult problem that is partly due to the formation of dipole-bound anions of these systems.[31] The ability of a molecule to stabilize an excess electron in a dipole-bound state depends primarily on the electrostatic interaction between the electron and the dipole moment of the neutral molecule. Generally, a dipole moment larger than 2.5 D is required to trap an electron in a dipole-bound state.[32] Dipole-bound anions of nucleic acid bases have been investigated both experimentally, e.g., using Rydberg electron transfer (RET) spectroscopy[31,33,34] and photodetachment−photoelectron spectroscopy (PD-PES),[35,36] and theoretically, using various ab initio methods.[31,37−40] With the use of diffuse functions in the basis sets and taking electron correlation effects into account, it was demonstrated that it is possible to obtain dipole-bound states also in the thymine radical anion.[31] The diffuse functions are necessary since the excess electron usually is delocalized outside the molecule along the positive direction of the molecular dipole. These calculations also showed that only dipole-bound thymine anions have energies lower than those in the neutral system.

In this work, the geometry optimizations of thymine and thymine dimer and their respective radical anion and cation forms were performed by using Becke's three-parameter hybrid exchange functional (B3)[41] in combination with the correlation functional of Lee, Yang, and Parr (LYP)[42] and the 6-31G(d,p) and 6-31+G(d) basis sets. Single-point calculations with the B3LYP functional and the 6-311++G(2df,p) basis set were performed on the global minima to obtain better relative energies and radical spin densities.

The optimized geometries of FADH$^-$, FADH$^•$, 8-HDF, and MTHF were determined at the B3LYP/6-31G(d,p) level. To obtain the energies of the first singlet excited states of FADH$^-$, 8-HDF, and MTHF, the implementation of the TDDFT method by Stratmann et al.[24] in the Gaussian 98 program[25] was employed. This implementation provides vertical excitation energies only, i.e. no geometry optimizations of the excited states are performed, and is, from a computational point of view, very demanding when applied to large molecules. Diffuse functions were for this reason omitted, and the single-point calculations on the respective ground-state geometries were carried out at the B3LYP/6-311G(2df,p) level. To make adequate comparisons, the absolute energies of the ground states of FADH$^-$, FADH$^•$, 8-HDF, and MTHF were also determined at the B3LYP/6-311G(2df,p) level.

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Table 1. Selected Bond Lengths and Torsion Angles (Å and deg, respectively) of Thymine Dimer and Its Radicals[a]

	B3LYP/6-31G(d,p)			B3LYP/6-31+G(d)			HF/6-31G[46]	
	T_2	$T_2^{\bullet-}$	$T_2^{\bullet+}$	T_2	$T_2^{\bullet-}$	$T_2^{\bullet+}$	T_2	$T_2^{\bullet+}$
bond lengths								
C5−C5′	1.593	2.761	1.602	1.597	2.807	1.606	1.590	1.599
C6−C6′	1.570	1.562	2.188	1.569	1.561	2.174	1.557	2.106
C4−C5	1.527	1.424	1.544	1.528	1.423	1.544	1.512	1.526
C4′-C5′	1.532	1.421	1.544	1.533	1.419	1.544	1.518	1.530
torsion angles								
C5−C5′−C6′−C6	19.4	21.8	18.2	19.1	22.7	17.4	17.9	14.7
C7−C5−C5′−C7′	27.6	30.0	30.2	27.1	32.4	28.5	25.2	23.0
H6−C6−C6′−H6′	25.5	41.8	22.1	25.0	44.0	21.0	23.5	17.9

[a] The corresponding data from a previous study[46] are given in the two columns to the right.

From the B3LYP/6-31+G(d) optimized structure of the thymine dimer radical anion (Figure 4 and Table 1), we see that the C6−C6′ distance is 1.56 Å and the C5−C5′ distance is 2.81 Å. Consequently, we do not expect a barrier in the cleavage of the C5−C5′ bond upon electron uptake of the dimer. The kinetics of the fragmentation of the C6−C6 bond, on the other hand, was investigated in the following way. Initially, partial B3LYP/3-21G geometry optimizations were performed with the C6−C6′ distance held fixed at different bond lengths ranging from 1.56 to 2.06 Å. The highest energy structure was then subject to a transition-state optimization at the B3LYP/6-31+G(d) level. Finally, a single-point calculation at the B3LYP/6-311++G(2df,p) level was performed on the transition-state optimized structure.

At the level of theory used in the present study calculating zero-point vibrational energy corrections for the different thymine dimers and cofactors is a very demanding task. However, initial calculations at the B3LYP/6-31G level (geometry optimizations and subsequent frequency analyses) showed that these corrections are small in terms of relative energies and will not change any of the conclusions to be drawn in this study. The zero-points effects were for this reason not taken into account at the higher levels of theory. All stationary points obtained at the B3LYP/6-31G level displayed the correct curvature. As inclusion of polarization and diffuse functions in the basis set was shown to imply only small structural changes in the optimized geometries (typically a few thousandths of an angstrom for the bond lengths and a few degrees for the torsion angles); we believe that this is not a crude approximation.

The direction of the electron transfer in the photoreactivation process, i.e. the transfer of one electron from a singlet excited $FADH^-$ to the dimer, is, as previously mentioned, deduced from thermodynamic arguments and isotope effects studies. According to Heelis et al.[16,18] $\Delta G = -29.9$ kcal mol^{-1} for electron transfer to the dimer, whereas $\Delta G = +43.1$ kcal mol^{-1} is required for electron transfer from the dimer (to the singlet excited $FADH^-$). These results would exclude an alternative reaction pathway involving the formation of a thymine dimer radical cation ($T_2^{\bullet+}$) and a two-electron reduced flavin species ($FADH^{\bullet 2-}$). However, since the reported energies could be marred by errors,[16] we have also investigated the thermodynamics of the alternative electron transfer. It should be stressed that $FADH^{\bullet 2-}$ has been observed in pulse radiolysis studies,[43] and that it has spectral properties similar to those detected after photoexcitation of the enzyme−dimer complex.[16] Furthermore, Aida et al.[44] have shown that the thymine dimer radical cation is easily fragmented. In other words, the proposed mechanism of the photoreactivation process is not entirely undisputed.

Results and Discussion

Energies of Enzymatic Cofactors. The relative energies of the different states of $FADH^-$, 8-HDF, and MTHF are presented in Table 2. The vertical singlet excitation energies of the cofactors are in excellent ($FADH^-$ and MTHF), or good (8-HDF), agreement with experimental data.[2,16,18] This suggests that our model cofactors are reasonable and that electronic transition energies of large organic molecules can be accurately determined by the TDDFT procedure. In this study, the overall tendency is that the method provides energies that are somewhat higher than the experimental ones.

Table 2. Relative Energies (kcal mol^{-1}) of Enzymatic Cofactors in Different States

cofactor	B3LYP/6-311G(2df,p)// B3LYP/6-31G(d,p)	exptl value[2,16]
$FADH^-$	0	0
$FADH^{\bullet}$	42.9	
$^1FADH^-$	61.1	57.3
$FADH^{\bullet 2-}$	96.9	
8-HDF	0	0
^{1}8-HDF	77.3	68.1
MTHF	0	0
^{1}MTHF	76.3	73.3

Table 3. Relative Energies (kcal mol^{-1}) for Various Thymine Derivatives

	B3LYP/6-311++G(2df,p)// B3LYP/6-31G(d,p)	B3LYP/6-311++G(2df,p)// B3LYP/6-31+G(d)
T	0	0
$T^{\bullet-}$	0.07	−0.59
$T^{\bullet+}$	202.6	202.6
T_2	0	0
$T_2^{\bullet-}$	−20.2	−20.3
$T_2^{\bullet+}$	196.0	196.0

We also note that $FADH^{\bullet}$ is 18.2 kcal mol^{-1} lower, and $FADH^{\bullet 2-}$ 35.8 kcal mol^{-1} higher, in energy than $^1FADH^-$.

Structures and Energies of Thymine Derivatives. The relative energies of the different forms (neutral, radical anion, and radical cation) of thymine and thymine dimer are listed in Table 3. We observe that the adiabatic electron affinity (EA) of thymine is positive (EA = 0.59 kcal mol^{-1}) at the B3LYP/6-311++G(2df,p)//B3LYP/6-31+G(d) level and negative (EA = −0.07 kcal mol^{-1}) at the B3LYP/6-311++G(2df,p)//B3LYP/6-31G(d,p) level. The effect of using diffuse functions in the optimizations is thus apparent. The former result is in fairly close agreement with several experimental estimates of the dipole-bound electron affinity (EA_{db}) of thymine. From PD-PES, Bowen and co-workers and Neumark and co-workers have obtained $EA_{db} \approx 1.6$ kcal mol^{-1} (ref 35) and $EA_{db} \approx 1.4$ kcal mol^{-1} (ref 36), respectively. Using RET spectroscopy, Schermann and co-workers have reported $EA_{db} \approx 1.6$ kcal mol^{-1}.[33] EA = 0.59 kcal mol^{-1} also agrees well with the ab initio result $EA_{db} \approx 0.73$ kcal mol^{-1} of Adamowicz and co-workers.[31] The B3LYP/6-311++G(2df,p)//B3LYP/6-31+G(d) thymine dipole moment (4.5 D) is in accordance with previous investigations (Adamowicz et al., 4.9 D;[31] Jernigan et al., 4.6 D[45]). The adiabatic electron affinity of the dimer, in turn, is roughly 20

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kcal mol^{-1} in both calculations. When diffuse functions are included in the optimization, the dipole moment is sufficiently high (7.0 D) to ensure the existence of a dipole-bound state. The data in Table 3 also show that the diffuse functions have no effect on the adiabatic ionization potentials (IPs) of the monomer and the dimer ($IP_{monomer}$ = 202.6 kcal mol^{-1} and IP_{dimer} = 196.0 kcal mol^{-1} in both calculations).

The geometry-optimized structures of thymine dimer and its radical anion and cation forms are depicted in Figure 4, and some of the key geometric parameters are listed in Table 1. We observe that the differences between B3LYP/6-31G(d,p) and B3LYP/6-31+G(d) parameters are generally small, and we will here discuss the latter ones. The distance between C5 and C5′ increases from 1.60 to 2.81 Å when one electron is added to the dimer. This indicates that the cleavage of the C5−C5′ bond is the first step in the fragmentation of the dimer. No transition state is located for the breakage of this bond. The C4−C5 and C4′−C5′ bond lengths of the dimer radical anion are roughly 0.11 Å shorter than those in the neutral dimer, i.e. the C4−C5 bonds start to display double-bond character upon electron addition.

Removal of one electron from the dimer implies a substantial lengthening of the C6−C6′ bond (from 1.57 to 2.17 Å). Hence, fragmentation of a thymine dimer according to the alternative cationic reaction pathway would start with the cleavage of the C6−C6′ bond.

Table 1 also includes parameters from an ab initio MO study on the dimer and its radical cation.[46] As can be seen, the agreement is generally good with somewhat larger discrepancies between the torsion angles of the radical cation.

If we now consider the catalytic cofactor and the dimer as being an isolated system, i.e. we neglect the impact of the surrounding enzyme on the energetics of this system, the electron transfer from $^1FADH^-$ to T_2 is, recalling the energies in Tables 2 and 3 (B3LYP/6-311++G(2df,p)//B3LYP/6-31+G(d) energies in Table 3), exothermic by 38.5 kcal mol^{-1} while the reverse reaction is endothermic by 231.8 kcal mol^{-1}. To be able to compare our calculated energies with availble experimental data, we will in the following disregard thermal corrections to the electronic energies and denote, somewhat improperly, $\Delta E^{DFT} = \Delta G^{DFT}$. Some justification for this simplification is presented in a study by Rösch et al.[47] They argue that the translational and rotational degrees of freedom do not significantly contribute to the reaction free energy in a biologically relevant DNA−enzyme complex (a bound system). Furthermore, the vibrational entropy should be small. As an example, they show that the entropy contribution to the splitting of a uracil dimer radical anion decreases from −13 kcal mol^{-1} (for a free molecule in the gas phase) to approximately −3 kcal mol^{-1} when the dimer is bridged by a trimethylene group in a bound system. With this simplification we have:

$$^1FADH^- + T_2 \xrightarrow{\Delta G^{DFT} = -38.5 \text{ kcal mol}^{-1}} FADH^\bullet + T_2^{\bullet -} \quad (1)$$

$$^1FADH^- + T_2 \xrightarrow{\Delta G^{DFT} = +231.8 \text{ kcal mol}^{-1}} FADH^{\bullet 2-} + T_2^{\bullet +} \quad (2)$$

A comparison with the energies presented by Heelis et al.[16,18] shows that our theoretical approach provides a fairly similar description of the thermodynamics of the electron transfer from the catalytic cofactor to the dimer (ΔG^{DFT} = −38.5 kcal mol^{-1} vs ΔG^{exp} = −29.9 kcal mol^{-1}). The value of ΔG^{exp} was basically calculated from experimental data on the difference in reduction potential between donor ($^1FADH^-$) and acceptor (T_2) involved in the electron-transfer process,[19] and serves as one of the major arguments for the proposed reaction mechanism. However, estimates of free-energy changes according to this approach could be uncertain.[16] Since we are able to verify the exothermicity of this step, we can conclude that our calculations support the proposed anionic reaction mechanism. The calculated thermodynamics of electron transfer in the opposite direction, on the other hand, compares poorly with the corresponding experimental energy (ΔG^{DFT} = +231.8 kcal mol^{-1} vs ΔG^{exp} = +43.1 kcal mol^{-1}). Apparently, our calculations suggest a much larger difference in free-energy change, $\Delta\Delta G$, between the two possible directions of electron transfer ($\Delta\Delta G^{DFT}$ = 270.3 kcal mol^{-1} vs $\Delta\Delta G^{exp}$ = 73.0 kcal mol^{-1}). This discrepancy is, obviously, significant. However, given that the calculated IP = 196.0 kcal mol^{-1} is in excellent agreement with previous studies,[46] and the electron affinity of $^1FADH^-$ is endothermic (or, at the best, thermoneutral based on previous work by us and others[48−50]), one would expect the free-energy change of the alternative electron transfer to be highly endothermic. We therefore believe that our result is trustworthy.

Thermodynamics of the Photoreactivation Process. We have, molecule by molecule, calculated the energies of the reactants, the intermediates, and the products of the photoreactivation process. If we assume that the interactions between the enzyme and the different molecules are weak, these energies should be able to at least qualitatively describe the thermodynamics of the photoreactivation process. We know from the crystallographic structures[14,15] that the cofactors are separated by 17.5 (8-HDF and $FADH^-$) and 16.8 Å (MTHF and $FADH^-$), respectively. We can, consequently, ignore any other cofactor−cofactor interaction besides the actual transfer of excitation energy. Furthermore, we consider two thymine residues in the same strand of a DNA molecule as essentially noninteracting as they are separated by roughly 4 Å in their stacked arrangement in B DNA.[51] The strength of the interaction between the thymine dimer and the catalytic cofactor is more difficult to predict since, to date, no structure of an enzyme−substrate complex has been determined. Park et al.[14] have speculated that the dimer would be in van der Waals contact with the isoalloxazine ring of $FADH^-$, and that this mode of binding would enable efficient electron transfer to the dimer. Support for this speculation is provided in an earlier study by Kim et al.[52] showing that one *Escherichia coli* DNA photolyase tryptophan residue (Trp-277), which is involved in substrate binding, can repair a thymine dimer directly with a high quantum yield. On the contrary, in a molecular dynamics simulation of an enzyme−substrate complex,[53] no close contacts between the dimer and the catalytic cofactor were displayed; the smallest distance between the dimer and the isoalloxazine ring of $FADH^-$ was approximately 10 Å throughout the data collection phase. This simulation, however, focused on an initial enzyme−substrate complex, i.e., the conformational changes of the

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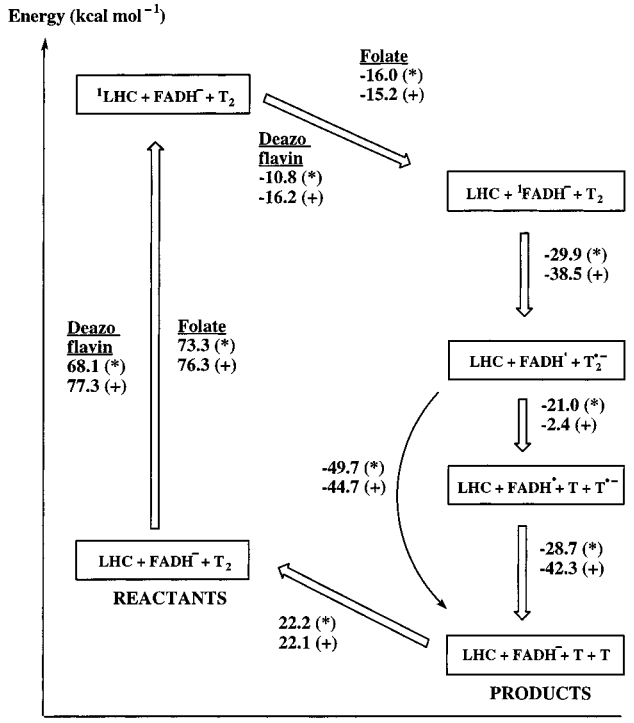

Figure 5. The thermodynamics of the major steps involved in the photoreactivation process catalyzed by a DNA photolyase: LHC = deazoflavin (8-HDF) or folate (MTHF); (*) from experiment;[2,16,18] and (+) absolute energies of $FADH^-$, $^1FADH^-$, $FADH^\bullet$, LHC and ^{1}LHC from B3LYP/6-311G(2df,p)//B3LYP/6-31G(d,p), absolute energies of T, $T^{\bullet-}$, $T^{\bullet+}$, T_2, $T_2^{\bullet-}$, and $T_2^{\bullet+}$ from B3LYP/6-311++ G(2df,p)//B3LYP/6-31+G(d).

complex as the photoreactivation process proceeds were not described in the study. In the present work, we will assume that any contact between the dimer and the catalytic cofactor is weak (negligible).

The overall calculated thermodynamics according to these simplifications are outlined in Figure 5 together with the corresponding experimental energies.[16,18] The calculated energy changes of the two initial steps, excitation of the light-harvesting cofactor and energy transfer to the catalytic cofactor, are, as we have already seen, in good agreement with experimental data for both classes of enzymes. The energy transfer step is, quite naturally, exothermic since we would not expect the transfer to occur with 100% efficiency, i.e., without any loss of excitation energy from the system. The subsequent steps,; electron transfer from $^1FADH^-$ to the dimer, splitting of the dimer radical anion, and back electron transfer to the FADH-radical, are the key steps of the photoreactivation process. As noted above, our DFT studies and previous semiexperimental studies[16,18] provide fairly similar descriptions of the thermodynamics of the initial electron transfer. But the different approaches do not assign similar thermodynamics to the splitting of the dimer radical anion and the back-electron transfer. According to the calculations, the splitting is only slightly exothermic ($\Delta G^{DFT} = -2.4$ kcal mol^{-1}) whereas the experimental energies propose $\Delta G^{exp} = -21.0$ kcal mol^{-1}. In addition, the calculations assign a higher exothermicity to the back-electron transfer ($\Delta G^{DFT} = -42.3$ kcal mol^{-1} vs $\Delta G^{exp} = -28.7$ kcal mol^{-1}). These discrepancies are discussed in more detail below. However, the descriptions of the thermodynamics of these two steps *added together* show a similar exothermicity ($\Delta G^{DFT} = -44.7$ kcal mol^{-1} vs $\Delta G^{exp} = -49.7$ kcal mol^{-1}). Taking into account that our calculations also suggest that the overall reaction is exothermic by 22.1 kcal mol^{-1} (cf. $\Delta G^{exp} = -22.2$ kcal mol^{-1}), we claim that our calculations constitute a thermodynamical support for the proposed anionic reaction mechanism.

The experimental free-energy change of the back-electron transfer step is easily derived once the difference in one-electron reduction potential between donor (**E**[T/T$^{\bullet-}$]) and acceptor (**E**[FADH$^\bullet$/FADH$^-$]) has been determined (see eq 3, Heelis et al.[16]). The experimental free-energy change of the splitting of the dimer is, however, more problematic. In this case, the difference in one-electron reduction potential between dimer (**E**[T_2/$T_2^{\bullet-}$]) and monomer (**E**[T/T$^{\bullet-}$]) has to be determined. Obviously, the experimental thermodynamics of the back-electron transfer and the fragmentation are not independent since both estimates rely on the adequacy of **E**[T/T$^{\bullet-}$]. Furthermore, if **E**[T/T$^{\bullet-}$] is overestimated, the exothermicity of the fragmentation will be overestimated and the exothermicity of the back-electron transfer underestimated equally as much. Hence, the overall thermodynamics of the fragmentation and the back-electron-transfer steps added together are independent of **E**[T/T$^{\bullet-}$]. Since our DFT calculations provide more or less the same overall thermodynamics, it is possible that the discrepancies between theoretical and experimental energies could be due to an experimental overestimate of the reduction potential of the monomer. This could explain why the experimental approach assigns a higher exothermicity to the fragmentation and a lower exothermicity to the back-electron transfer. One must not, however, forget that we are neglecting the (presumably small) thermal corrections to the calculated reaction energies of the fragmentation.[47] Some support for the thermodynamics obtained by Heelis et al.[16,18] is presented in a study by Falvey and co-workers, in which the enthalpy of cleavage of a *cis-syn* 1,3-dimethylthymine dimer radical anion is estimated.[54] Using photothermal beam deflection calorimetry, $\Delta H = -28$ kcal mol^{-1} is reported.

If the calculated thermodynamics of the splitting of the dimer radical anion is accurate (eq 3), i.e. if the splitting is only slightly exothermic, one might ask why, keeping in mind that our calculations as well as experiments suggest that the corresponding fragmentation of a neutral dimer into two neutral thymines (eq 4) would be exothermic by roughly 22 kcal mol^{-1}, evolution has favored an anionic reaction mechanism as opposed to a neutral?

$$T_2^{\bullet-} \xrightarrow{\Delta G^{DFT} = -2.4\ \Delta G^{exp} = -21.0\ \text{kcal mol}^{-1}} T + T^{\bullet-} \qquad (3)$$

$$T_2 \xrightarrow{\Delta G^{DFT} = -22.1\ \Delta G^{exp} = -22.2\ \text{kcal mol}^{-1}} T + T \qquad (4)$$

According to the Woodward−Hoffmann rules, a thermally induced fragmentation of a neutral dimer takes place only under extreme conditions, i.e., the process requires a substantial amount of activation energy. Hence, an estimate of the energy barrier(s) in the splitting of the dimer radical anion would address this question.

The fragmentation of a dimer radical *cation* has been investigated theoretically by Aida et al.[44] The energies presented in their work predict an overall 29.4 kcal mol^{-1} exothermic process and low-energy barriers in the splitting of the C5−C5′ and C6−C6′ dimer radical cation bonds (1.4 and 0.3 kcal mol^{-1} respectively). However, we claim that a cationic reaction mechanism is improbable due to the very high endothermicity ($\Delta G^{DFT} = 231.8$ kcal mol^{-1}) of the initial electron transfer from the neutral dimer to $^1FADH^-$.

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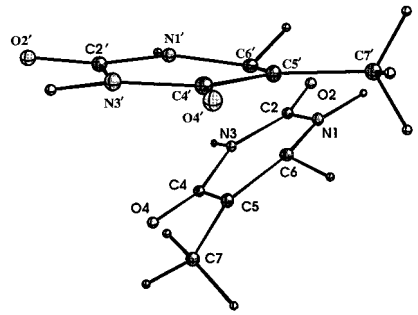
Kinetics of the Splitting of the Thymine Dimer Radical Anion. The splitting of pyrimidine dimer radical anions have previously been studied by Rösch et al.[28,47,55] In an early work,[47] a two-step mechanism was proposed based on AM1 and HF/6-31G(d) optimizations. After electron capture, they were able to locate a transition state with a barrier of 4.7 kcal mol^{-1} for the initial rupture of the C5−C5′ bond in $T_2^{\bullet-}$ (3.9 kcal mol^{-1} for the uracil dimer radical anion, $U_2^{\bullet-}$), followed by a ring-opened intermediate 18.5 kcal mol^{-1} below the cyclic reactant (−7.5 kcal mol^{-1} for $U_2^{\bullet-}$). Cleavage of the second bond, C6−C6′, was associated with a barrier of 5.3 kcal mol^{-1}, and the final T + $T^{\bullet-}$ product was exothermic by 30.6 kcal mol^{-1} relative to the isolated reactants. For $U_2^{\bullet-}$, the corresponding second barrier and overall reaction energy were 5.8 and −21.1 kcal mol^{-1}, respectively. In a subsequent study, the $U_2^{\bullet-}$ system was reevaluated using single-point MP2/6-31G(d) calculations on HF optimized structures.[55] It was then found that the first barrier (splitting of the C5−C5′ bond) vanished, and that the overall process was *endothermic* by 16.2 kcal mol^{-1}. In addition, no transition state could be located for the cleavage of the second C6−C6′ bond at this level of theory.

It is quite obvious from the methods employed and results reported that, first of all, insufficient basis sets were used. If nothing else, the computed uracil electron affinitity[55] (−1.20 eV vs +0.2 eV at the B3LYP/6-311+G(2df,p)//B3LYP/6-31+G(d,p) level[56]) is a clear indication of this. When investigating pyrimidine radical anions, diffuse functions are essential for accurate geometries as well as energetics.[56] Second, HF and MP2 theories are notoriously known to have problems with spin contamination when applied to radical systems.[57,58] Spin contamination effects are well established to provide additional minima, incorrect transition states, and energetics that do not correlate with observed reaction rates. Hence, pure HF data are simply not trustworthy for radical systems, and MP2 data cannot be reported without inclusion of spin projection techniques. Even then, optimized geometries should be considered with caution.

In the present work, using B3LYP/6-311++G(2df,p) energies on B3LYP/6-31+G(d) optimized structures, no initial transition state could be located (in agreement with the most correlated data set by Rösch et al.[55]). The initial ring-opening process will, hence, occur spontaneously upon electron uptake by the thymine dimer, and we will have a one-step fragmentation process. Interestingly, this result contradicts previous experimental investigations of a stepwise, as well as a concerted, mechanism that suggest that the C5−C5′ bond cleavage is part of the rate determining step.[22,59] For the cleavage of the C6−C6′ bond, a transition state was located. The activation energy for this process is 2.3 kcal mol^{-1}, and the T + $T^{\bullet-}$ product lies 2.4 kcal mol^{-1} below the ring-opened intermediate. The smaller barrier and lower exothermicity for the process compared with the AM1 data of Rösch et al.[47] is in accord with the general tendency of semiempirical and HF methods to generate too corrugated energy surfaces.

Rösch and co-workers also investigated the effects of protonation on the cleavage of $U_2^{\bullet-}$, this time using B3LYP/6-31G(d) and AM1 levels of theory.[28] The conclusion from their investigation was that protonation is energetically unfavorable, and that it furthermore yields slower overall reaction rates due to higher barriers.



C5-C5′ = 3.518
C6-C6′ = 2.025
C5-C5′-C6′-C6 = 26.7
C7-C5-C5′-C7′ = 74.2
H6-C6-C6′-H6′ = 71.6

Figure 6. Transition state structure in the fragmentation of a thymine dimer radical anion. Numerical values (in Å and deg) are given for selected bond lengths and torsion angles.

Figure 6 shows the optimized transition state structure for the C6−C6′ bond breakage. Some of the key geometric parameters are also listed. We note that the C6−C6′ bond length is 2.03 Å, and that the major structural difference with respect to $T_2^{\bullet-}$ is the increase of the C7−C5−C5′−C7′ and H6−C6−C6′−H6′ torsion angles from 32.4° and 44.0° to 74.2° and 71.6°, respectively. The activation energy in the fragmentation is, as noted above, 2.3 kcal mol^{-1}. Once formed, $T^{\bullet-}$ will according to the calculations transfer its excess electron back to $FADH^{\bullet}$ in a highly exothermic process ($\Delta G^{DFT} = -42.3$ kcal mol^{-1}). The low-energy barrier to rupture of $T_2^{\bullet-}$ and the associated high exothermicity of back-electron transfer could explain why nature has chosen an anionic reaction mechanism.

Conclusions

In the present study, we show that density functional theory calculations support the proposed anionic reaction mechanism of the DNA photoreactivation process from a thermodynamical point of view. The electron transfer steps, as well as the overall reaction, are exothermic and the reaction energies assigned to the different steps are generally in good agreement with experimental data. We also show that the splitting of the thymine dimer radical anion is kinetically favored. Furthermore, on thermodynamic grounds, we believe that the alternative reaction pathway involving electron transfer *from*, rather than *to*, the neutral dimer is improbable. The excellent agreement between the experimental singlet excitation energies of the cofactors and the corresponding energies obtained by TDDFT indicates that this method is a promising tool to be used in future calculations of electronic transition energies in large bioorganic molecules.

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