Substrate Binding to DNA Photolyase Studied by Electron Paramagnetic Resonance Spectroscopy

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ABSTRACT Structural changes in *Escherichia coli* DNA photolyase induced by binding of a (*cis*,*syn*)-cyclobutane pyrimidine dimer (CPD) are studied by continuous-wave electron paramagnetic resonance and electron-nuclear double resonance spectroscopies, using the flavin adenine dinucleotide (FAD) cofactor in its neutral radical form as a naturally occurring electron spin probe. The electron paramagnetic resonance/electron-nuclear double resonance spectral changes are consistent with a large distance ($\geq$0.6 nm) between the CPD lesion and the 7,8-dimethyl isoalloxazine ring of FAD, as was predicted by recent model calculations on photolyase enzyme-substrate complexes. Small shifts of the isotropic proton hyperfine coupling constants within the FAD's isoalloxazine moiety can be understood in terms of the cofactor binding site becoming more nonpolar because of the displacement of water molecules upon CPD docking to the enzyme. Molecular orbital calculations of hyperfine couplings using density functional theory, in conjunction with an isodensity polarized continuum model, are presented to rationalize these shifts in terms of the changed polarity of the medium surrounding the FAD cofactor.

INTRODUCTION

DNA photolyases are flavoenzymes that catalyze the photorepair of (*cis*,*syn*)-cyclobutane pyrimidine dimers (CPDs) formed as lesion in UV-irradiated ($\lambda \leq 300$ nm) cellular DNA from two adjacent pyrimidine bases (Kim and Sancar, 1993; Sancar, 1994, 1996; Heelis et al., 1995, 1996; Zhao and Mu, 1998; Todo, 1999; Deisenhofer, 2000). The enzyme binds to the damaged part of the DNA strand in a light-independent step (Sancar et al., 1985; Kim and Sancar, 1991) and catalyzes its repair when exposed to near-UV or visible light (300 nm $< \lambda <$ 500 nm). The initial step in the repair pathway was proposed to be a photoinduced single electron transfer from the redox-active flavin adenine dinucleotide (FAD) cofactor in its fully reduced form, $FADH^-$ (Sancar et al., 1987; Payne et al., 1987), to the CPD lesion (Jordan and Jorns, 1988; Witmer et al., 1989; Okamura et al., 1991; Chanderkar and Jorns, 1991; Kim et al., 1991). As a consequence of this electron transfer reaction, the two bonds between the pyrimidine bases break in a [2 + 2] cycloreversion. Subsequently, the electron is transferred back to the flavin and the enzyme-CPD complex dissociates.

The understanding of the DNA repair process on the molecular level was greatly enhanced through the determination of the three-dimensional x-ray structures of the DNA photolyases from two organisms, *Escherichia coli* (Park et al., 1995) and *Anacystis nidulans* (Tamada et al., 1997). It was found that the FAD chromophore lies deeply buried in the center of a helical domain, and direct access of damaged DNA substrate is limited to a "hole," i.e., cavity entrance, leading from the edge of the 7,8-dimethyl isoalloxazine ring of FAD to the enzyme surface. A band of positive electrostatic potential, suitable for contact with the phosphate backbone of the DNA strand, runs along the outside of the protein around the cavity entrance. Its opening is wide enough to host a CPD, when flipped outside a DNA double helix (Park et al., 1995). Experimental evidence for dinucleotide flipping induced by DNA photolyase was presented recently, based on measured quantum yields and substrate association constants which were determined for a series of point mutations at amino acid residues that are believed to play a role in substrate binding and discrimination (Vande Berg and Sancar, 1998). It was predicted that one of the tryptophan residues (W277 in *E. coli* DNA photolyase) adjacent to the FAD cofactor is in van der Waals contact with the DNA lesion, thus facilitating rapid electron transfer from the redox-active 7,8-dimethyl isoalloxazine moiety to the CPD.

To unravel the mechanism of this light-induced electron transfer step, be it via intermediate sequential electron acceptors, via superexchange, or a combination of both, requires knowledge about the distance between the redox partners and their relative orientation. To date, no photolyase enzyme-substrate cocrystals exist for x-ray structure determination and, therefore, information on their relative geometry relies on modeling. Several model calculations on substrate binding to DNA photolyase have been presented recently (Vande Berg and Sancar, 1998; Sanders and Wiest, 1999; Hahn et al., 1999; Antony et al., 2000). They were performed using docking procedures of various kinds to fit the dimer lesion into the substrate binding pocket, followed by energy minimizations to equilibrate the assembly structures. The results of these computations, however, are in-

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consistent. In two of the three molecular dynamics studies the minimum distance between the flavin cofactor and the basepair containing the CPD is $\geq$0.6 nm when model dinucleotides and related derivatives were examined (Sanders and Wiest, 1999; Hahn et al., 1999). Substantially wider distances are predicted when single-strand or duplex oligonucleotides containing a CPD lesion were studied (Sanders and Wiest, 1999). In these more realistic systems the nonpolar CPD moiety of the dimer is at least 1 nm distant from the FAD's 7,8-dimethyl isoalloxazine moiety. Contrary to these findings, the third molecular dynamics study predicts a very short separation of $<$0.3 nm for the substrate-cofactor distance (Antony et al., 2000). In the fourth model study, neither coordinates nor distances are presented (Vande Berg and Sancar, 1998); however, as can be inferred from Fig. 6, shorter (vs. longer) FAD cofactor-to-substrate distances are preferred.

In the present study, electron paramagnetic resonance (EPR) and electron-nuclear double resonance (ENDOR) experiments are performed to present experimental evidence that allows an assessment of the various theoretical models presented for enzyme-substrate binding in DNA photolyase. In the *E. coli* photolyase the redox-active FAD cofactor is reversibly transformed into its neutral radical form, $FADH^{\bullet}$, upon isolation and purification of the protein in an aerobic environment (Jorns et al., 1984, 1990; Payne et al., 1987). The $FADH^{\bullet}$ radical thus produced can be viewed as a naturally occurring spin probe at the active site of the enzyme. Proton hyperfine couplings (hfcs) have been measured and assigned in a recent EPR/ENDOR study on the isolated enzyme (Kay et al., 1999). A theoretical analysis on the electronic structure of the flavin cofactor in DNA photolyase has recently been provided (Weber et al., 2001). It was shown that environmental effects, such as hydrogen bonding, have a marked influence on the spin density distribution, and hence, on the hfcs within the 7,8-dimethyl isoalloxazine moiety of $FADH^{\bullet}$. As the DNA substrate binding to the enzyme does not depend on the redox state of the flavin cofactor (Sancar et al., 1987; Payne et al., 1990), a spin probe study of substrate binding is made possible by comparison of measured hfcs for the enzyme's $FADH^{\bullet}$ radical with and without bound substrate. Similar experiments have been performed previously on the substrate binding to other flavoenzymes, such as the cholesterol oxidase from *Brevibacterium sterolicum* (Medina et al., 1994). To substantiate our interpretation of the measured changes in the proton hfcs of $FADH^{\bullet}$ upon substrate binding, we present model calculations on the solvent dependence of flavin proton hfcs. They use the static isodensity surface polarized continuum model (IPCM; Foresman et al., 1996) in conjunction with density functional theory (DFT; Hohenberg and Kohn, 1964; Kohn and Sham, 1965; Parr and Yang, 1989; Koch and Holthausen, 2000).

MATERIALS AND METHODS

Preparation and isolation of DNA photolyase

All experiments presented in this contribution were performed on the E109A mutant of DNA photolyase from *E. coli*. Replacement of the glutamic acid E109 with alanine results in a light-harvesting cofactor (5,10-methenyltetrahydrofolylpolyglutamate)-devoid enzyme which, however, has the same DNA substrate binding and EPR/ENDOR spectral properties as the wild-type protein. Construction of the E109A mutant of DNA photolyase and its purification will be described elsewhere (Schleicher et al., manuscript in preparation). The enzyme concentration of 0.4 mM was calculated on the basis of $FADH^{\bullet}$ optical absorption at 580 nm ($\epsilon_{580} = 4800\ M^{-1}\ cm^{-1}$; Wang and Jorns, 1989). Absorption spectra were recorded at room temperature using a Shimadzu UV-1600PC (Shimadzu, Columbia, MD) spectrophotometer.

Preparation of substrate and monitoring of irradiation damage

An aqueous 4-mM solution of oligo$(dT)_{18}$ DNA was irradiated at 4°C for 45 min by UV radiation (254 nm) with a UV lamp (Sylvania, Danvers, MA) placed at a distance of 5 cm. The damage reaction was monitored photometrically (260 nm). Aliquots were retrieved at intervals and were analyzed by titration with $KMnO_4$ (Ramaiah et al., 1998). In brief, 2–10 μl of oligonucleotide solution, 6 μl of 1 M potassium phosphate, and 30 μl of 20 mM $KMnO_4$ were mixed, and water was added to a final volume of 600 μl in an inert gas atmosphere at room temperature. After 5 min, the reaction mixture was centrifuged at 10,000 $\times$ *g* for 1 min. UV/vis spectra between 460 and 590 nm were obtained from the supernatant. The integral was compared with reference spectra containing no oligonucleotide. The consumption of $KMnO_4$ is equivalent to undamaged thymidine.

EPR sample preparation

Sample preparations have been performed in an argon inert gas atmosphere under low-light conditions (red light). For optimum comparability of the effect of substrate binding, two samples were prepared from the same stock solution of DNA photolyase in a buffer (0.05 M HEPES, pH 7) containing 50% (v/v) glycerol: Sample A was prepared from 200 μl enzyme solution. UV-irradiated oligo$(dT)_{18}$ DNA in aqueous solution was added to yield a CPD-to-enzyme ratio of 5:1. With this large excess of CPD lesions, it is safe to assume that all enzyme molecules are bound to substrate. Sample B was prepared accordingly; however, instead of the aqueous substrate solution, an equivalent amount of water was added to have identical enzyme concentration and solvent viscosity of both samples. After preparation both samples were frozen and kept in liquid nitrogen in the dark.

Assay of DNA photolyase activity

The repair activity of the DNA photolyase enzyme was monitored by the recovery of the absorbance at 260 nm (arising from repaired thymidine) at intervals (1–20 min) as a result of the exposure of the sample to light irradiation in a 2-mm quartz cuvette using a slide projector.

EPR/ENDOR instrumentation

Continuous-wave (cw) EPR spectra at X-band frequencies (9–10 GHz) were obtained using a laboratory-built spectrometer. The main components are a Bruker ER041MR (Bruker, Rheinstetten, Germany) microwave bridge and a Varian E-9 (Varian, Palo Alto, CA) magnet. Samples were placed in a Bruker ER4118X-MD-5W1 dielectric resonator, which was immersed in a laboratory-built helium gas flow cryostat.

ENDOR spectra were recorded using a laboratory-built X-band spectrometer consisting of an electromagnet and a Bruker ER041MR microwave bridge, in conjunction with a Bruker EN801 ENDOR cavity. A radio-frequency (RF) synthesizer Hewlett-Packard 8660c (Hewlett Packard, Palo Alto, CA) in conjunction with a high-power RF amplifier ENI A-300 (ENI, Rochester, NY) was used to generate the cw RF field in the ENDOR cavity. Spectra were recorded at 160 K using a nitrogen gas flow adjusted by a Bruker ER4111VT temperature controller.

Model calculations

Solvation effects on proton hfcs of a flavin neutral radical were examined theoretically using the IPCM model (Foresman et al., 1996) in conjunction with DFT calculations at the B3LYP/6–31G* level of theory (Barone, 1995, 1996; Rega et al., 1996; Becke, 1993). For computational purposes the flavin radical was truncated at the C(3′) position (for the molecular numbering scheme, see Fig. 3). This cutoff position is well removed from the main π-electron system of the redox-active isoalloxazine ring and, therefore, the neglect of atoms beyond this point has a negligible effect on the electron spin density distribution of the free radical. The molecule was then energy minimized *in vacuo* using the same hybrid functional and basis set as were used subsequently for the computation of hfcs as a function of the solvent's dielectric constant, ϵ_r. All computations were performed using Gaussian 98 (Frisch et al., 1998) with tight self-consistent field convergence criteria.

RESULTS AND DISCUSSION

Before presenting the results of our EPR studies, it should be pointed out that DNA photolyases are structure-specific enzymes that display binding discrimination comparable with that seen from sequence-specific DNA-binding proteins. Studies on the *E. coli* and *Saccharomyces cerevisiae* photolyase enzymes have shown that the equilibrium association constant for (*cis,syn*)-CPDs in DNA is $\sim 10^9$ M^{-1}, whereas the association constant for undamaged DNA is only 10^3 M^{-1} (Sancar, 1990; Husain and Sancar, 1987; Baer and Sancar, 1993; Li and Sancar, 1990). (*trans,syn*)-CPDs bind to photolyase with an association constant of 10^4 M^{-1}. Although the quantum yield of DNA repair is considerably smaller in photolyase where the FAD is in the neutral radical form, $FADH^{\bullet}$ (compared with enzyme with the FAD in the fully reduced state, $FADH^-$), the redox state of the flavin does not alter CPD binding (Sancar et al., 1987; Payne et al., 1990). Thus, the blue radical form of the enzyme bound to substrate is not only a well suited intermediate analogue for studying transient states present during DNA repair (Kay et al., 1999; Weber et al., 2001) but also represents an ideal probe for the investigation of enzyme-substrate interactions in photolyases.

EPR spectroscopy

Fig. 1 depicts the almost symmetric cw X-band EPR signal of $FADH^{\bullet}$ observed at 150 K. The signal is centered at $g = 2.0034 \pm 0.0002$. In excellent agreement with values observed in previous studies (Kay et al., 1999; Gindt et al., 1999; Rustandi and Jorns, 1995), the peak-to-peak linewidth

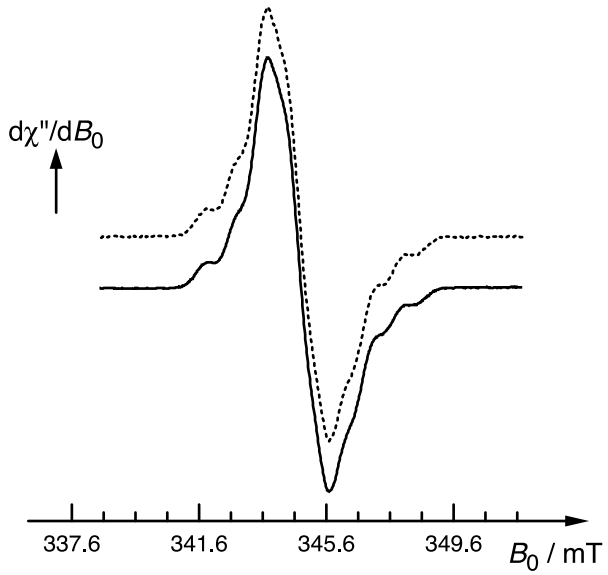

FIGURE 1 X-Band cw EPR frozen-solution spectra of the flavin neutral radical, $FADH^{\bullet}$, in DNA photolyase from *E. coli* without (*upper trace*, *dotted line*) and with (*lower trace*, *solid line*) added CPD substrate in H_2O. Spectra were recorded at 150 K with microwave power 3.0 μW, microwave frequency 9.6683 GHz, and modulation amplitude 0.05 mT (100 kHz field modulation).

of the signal is 1.87 mT. It exhibits some weakly resolved hyperfine structure with a spacing of ~0.85 mT between adjacent shoulders. This structure has been attributed to the isotropic hfc of the strongly coupled proton at the N(5) position (for the IUPAC numbering scheme, see Fig. 3) of the flavin cofactor's isoalloxazine moiety (Kay et al., 1999). Replacement of H(5) with deuterium upon exchanging the protonated for deuterated buffer results in a decrease in the peak-to-peak linewidth to a value of 1.30 mT (Kay et al., 1999). This is attributable to the smaller nuclear moment of a deuteron as compared with that of a proton. By contrast, the anionic form of the flavin cofactor, $FAD^{\bullet -}$, which has no strongly coupled exchangeable proton present at the N(5) position, would not be expected to show narrowing in D_2O as compared with H_2O (Kurreck et al., 1984; Medina et al., 1994; Çinkaya et al., 1997).

Preparation of the DNA photolyase enzyme in the presence of the CPD substrate containing photogenerated CPD lesions had almost no effect on the linewidth and the spacing of the partly resolved hyperfine splitting of the $FADH^{\bullet}$ signal. This is in stark contrast to a previous EPR study at 4 K in which an asymmetric 0.17-mT increase of the peak-to-peak EPR linewidth of $FADH^{\bullet}$ in *E. coli* DNA photolyase upon substrate binding was reported (Rustandi and Jorns, 1995). In our laboratory we have been unable to reproduce similar asymmetric EPR line broadening effects of $FADH^{\bullet}$ upon substrate binding when the experimental conditions were carefully chosen to avoid any EPR line distortions because of partial saturation or sample misalign-

ment (Poole, 1983). Therefore, the reported asymmetry probably arises from lineshape distortions because of unfavorable instrument or sample settings rather than changes of the $FADH^{\bullet}$ radical upon substrate binding.

As no major changes in the EPR signal of the enzyme-substrate complex were observed, we conclude that only minor effects on the electronic structure of the flavin cofactor occur when the CPD lesion approaches the FAD cofactor. Resulting minor changes of hfcs, however, might be obscured by inhomogeneous EPR line-broadening present in frozen flavin radicals. Therefore, ENDOR spectroscopy with its enhanced spectral resolution in the case of inhomogeneously broadened unresolved EPR lines was used to obtain more information on the electronic structure of the FAD cofactor in the DNA photolyase enzyme-substrate complex.

ENDOR spectroscopy

The X-band ENDOR spectrum of the $FADH^{\bullet}$ radical from *E. coli* DNA photolyase, recorded at the centerfield position of the EPR spectrum, exhibits a detailed hyperfine structure of the flavin semiquinone, as shown in Fig. 2. The frozen sample gives rise to a powder-type spectrum which is symmetric around the proton Larmor frequency, ν_H. Typical features of the ENDOR spectra of flavin semiquinones are resonances from the methyl protons at C(8α) which exhibit an axially symmetric tensorial pattern, and the proton at C(6) which shows a rhombic tensorial pattern from which, typically, only the central zero-crossing of the derivative lineshape is observed (Kurreck et al., 1984; Medina et al., 1994; Çinkaya et al., 1997; Kay et al., 1999). Other spectral features at $\nu_H \pm 5.7$ MHz and $\nu_H \pm 1.4$ MHz could be assigned to hfc components of the two magnetically inequivalent β-protons at C(1′). The ratio of their isotropic hfcs yields structural information on the orientation of the ribityl side chain relative to the isoalloxazine ring of $FADH^{\bullet}$ (Kay et al., 1999). A group of signals with hfcs in the so-called "matrix" region ($\nu_H - 1.25$ MHz $\leq \nu \leq \nu_H + 1.25$ MHz) is the sum of contributions from weakly coupled protons of solvent water, protons from the amino acid residues on the protein near the cofactor binding site, and protons at the C(7α), C(9), and N(3) in the isoalloxazine ring.

The matrix ENDOR signal

Analysis of the matrix ENDOR signals in protonated and deuterated buffer yields information concerning the exchangeability of protons, both in the constituent solvent and on the apoprotein at distances within approximately ≤ 0.6 nm (Hyde et al., 1968; Eriksson et al., 1970) from the isoalloxazine moiety of $FADH^{\bullet}$. If the amplitude of the matrix ENDOR signal centered at ν_H is scaled to the signals of the methyl protons at C(8α) (at $\nu_H \pm 3.75$ MHz), it is found that the matrix signal is decreased by an amount of ~15% in deuterated buffer (Kay et al., 1999). This rather small intensity drop demonstrates that the immediate surrounding of the flavin radical, i.e., within the range of matrix ENDOR (Hyde et al., 1968; Eriksson et al., 1970), is constituted partly by groups of the protein with exchangeable protons and, possibly, also water molecules. Conversely, the large remaining intensity of the matrix ENDOR signal in deuterated solvent shows that the immediate surrounding of $FADH^{\bullet}$ to a considerable fraction (~85%) consists of hydrophobic groups of the protein with nonexchangeable protons. A hydrophobic flavin binding site is consistent with results from optical absorption spectroscopy (Jorns et al., 1990; Chanderkar and Jorns, 1991), as the spectral properties of flavins also strongly depend on solvent polarity. It is also conceivable that some of the protons in the cofactor's surrounding are not readily accessible and, therefore, their exchange would require rather harsh protein treatment such as denaturing, subsequent deuteration, and finally reconstitution of the protein. This possibility, however, can be safely ruled out because, for example, the proton at N(5) is easily replaced with a deuteron upon exchanging the protonated buffer for deuterated buffer (see above). Additionally, good accessibility of the flavin cofactor is required for efficient enzyme function where short CPD substrate-flavin cofactor distances facilitate rapid electron transfer in the DNA repair process.

Changes in the matrix ENDOR region might also be observed when substrates are added. Such changes could be an indication of movements of the protein, insertion of molecules such as the substrate itself, or replacement of water protons. Upon CPD substrate binding to the *E. coli* DNA photolyase enzyme, however, no additional features are observed in the region $\nu_H - 1.25$ MHz $\leq \nu \leq \nu_H + 1.25$ MHz, see Fig. 2 *A*. Further, the matrix signal intensity is slightly decreased by $2.4 \pm 0.2\%$. These findings are clear experimental evidence that no purely dipolar hfcs arising from protons in the CPD substrate are observed in the ENDOR spectrum of $FADH^{\bullet}$. Therefore, we conclude that the CPD substrate does not approach the isoalloxazine moiety of $FADH^{\bullet}$ closer than to ~0.6 nm. The decrease in intensity of the matrix signal upon substrate binding may be an indication that water molecules are edged out the substrate binding pocket, as the bulky CPD lesion pushes its way into the opening at the surface of the enzyme (Park et al., 1995). This, however, should lead to altered hfcs of the proton positions in the FAD cofactor's isoalloxazine moiety as a consequence of the changing polarity of the cofactor surrounding when water molecules are displaced. Such changes are actually observed and will be discussed below.

Cofactor-substrate distances >0.6 nm are compatible with recent model calculations in which DNA photolyase-substrate complexes have been assembled using various docking procedures followed by molecular dynamics en-

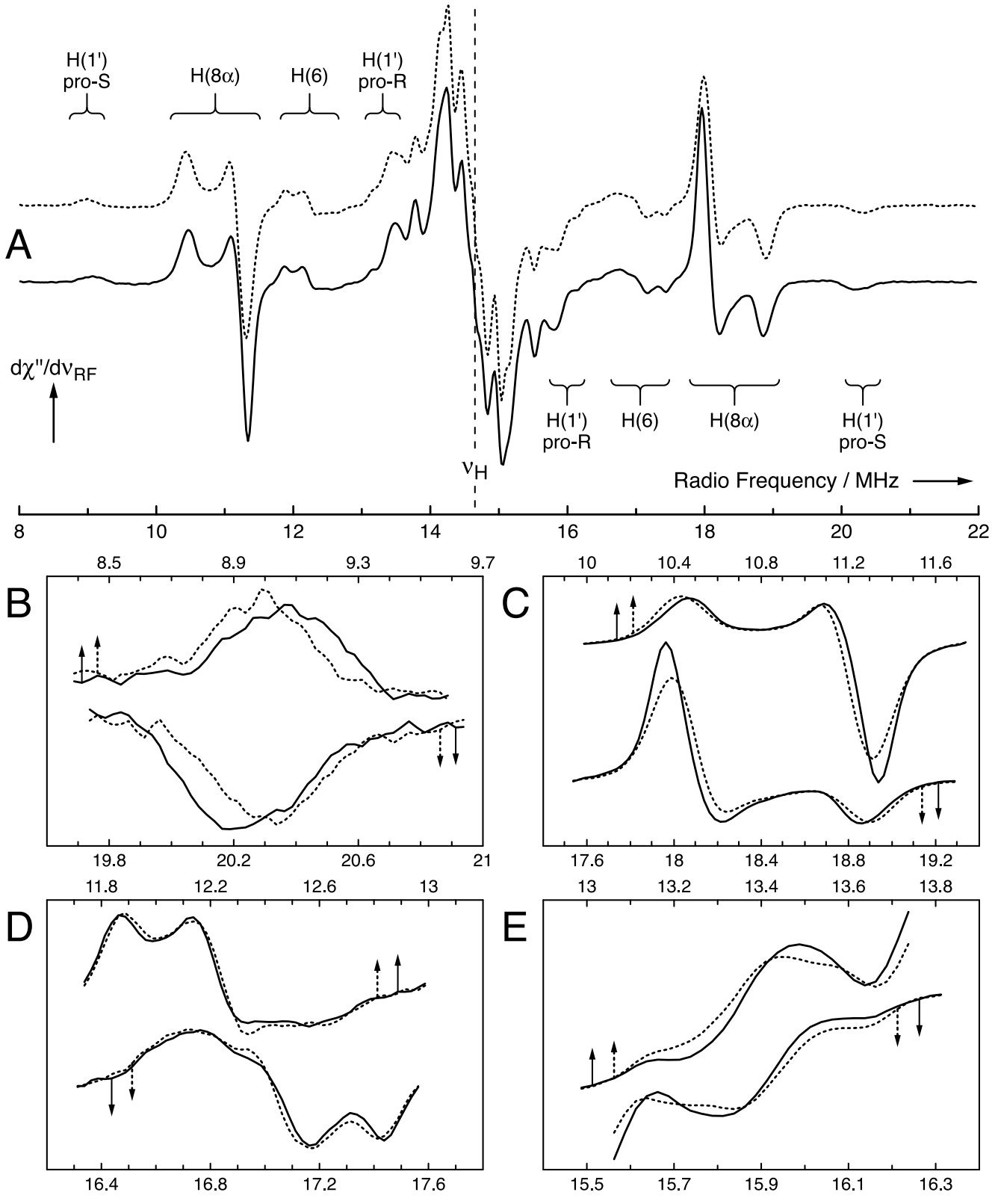

FIGURE 2 X-Band cw ENDOR frozen-solution spectra of the flavin neutral radical, $FADH^{\bullet}$, in DNA photolyase from *E. coli* without (*A*, *upper curve*, *dotted line*) and with (*A*, *lower curve*, *solid line*) bound CPD substrate. Spectra were recorded at 160 K with microwave power 2.5 mW, microwave frequency 9.6481 GHz, magnetic field 344.086 mT, RF modulation amplitude 40 kHz (19 kHz frequency modulation). The panels *B–E* show enlargements of various frequency ranges of the spectra of *panel A* for the enzyme without (*dotted lines*) and with (*solid lines*) bound CPD substrate. The arrows indicate the RF frequency axes to which the spectral traces belong.

ergy minimizations to equilibrate the aggregate geometries (Sanders and Wiest, 1999; Hahn et al., 1999). When the enzyme binding to pseudosubstrates consisting of one single damaged (*cis,syn*)-CPD segment (without backbone) was examined theoretically, Sanders and Wiest (1999) proposed that the smallest distance between the CPD lesion and the flavin cofactor is larger than 0.6 nm. Their results have subsequently been corroborated by Hahn and co-workers who have calculated a minimum distance of ~1.7 nm between one carbonyl oxygen in the CPD and N(1) of $FADH^{\bullet}$ (Hahn et al., 1999). Given such wide distances, only minor changes of the electronic structure of $FADH^{\bullet}$ are expected upon substrate binding. This is confirmed by the very similar EPR and proton ENDOR spectra for both the enzyme alone and the corresponding enzyme-substrate aggregate (Figs. 1 and 2 *A*).

In contrast to the aforementioned model calculations, a very short cofactor-substrate distance of <0.3 nm has been predicted from a very recent theoretical study (Antony et al., 2000). It was concluded that the CPD lesion approaches the redox-active isoalloxazine moiety of FAD from the "open" side between the ribityl side chain and the methyl group at C(8), establishing close contact between C(8α) and the methyl group of the CPD (distance ~0.25 nm). At such a short distance, not only additional, purely dipolar proton hfcs originating from the CPD substrate ought to appear in the matrix ENDOR region, but also the hfcs arising from protons within the isoalloxazine moiety would be expected to be considerably modified. Both effects have indeed been observed in earlier EPR/ENDOR studies on other flavoenzymes such as the cholesterol oxidase from *B. sterolicum*, where the steroid substrate (dehydroisoandrosterone) approaches the isoalloxazine moiety of the flavin cofactor (although from the N(5) side) to <0.3 nm (Li et al., 1993; Medina et al., 1994). Specifically, the hfc originating from the methyl protons at C(8α) is reduced upon substrate binding by almost 10% of its isotropic value when compared with the coupling in the absence of substrate. Such pronounced effects are, however, not observed in the EPR/ENDOR spectra of DNA photolyase presented here. This is why such a close encounter of the CPD lesion and the isoalloxazine ring in DNA photolyase, as proposed by Antony et al. (2000), seems to be rather unlikely. Further, a UV-damaged single-stranded DNA substrate consisting of 18 linked thymidine nucleotides was used in our work as a realistic model of a DNA strand, rather than single CPD fragments examined in the theoretical studies. For such DNA substrates, even wider distances between the CPD lesion and the FAD cofactor are expected because of the steric hindrance of the DNA backbone (Sanders and Wiest, 1999).

The hfcs from H(1′), H(6), and H(8α)

Upon binding of the CPD substrate to the photolyase enzyme, subtle changes are ENDOR-detected in the hfcs from the protons (Fig. 2 *B–E*). In particular, shifts of resonance frequencies are observed, but no line-broadening effects. The latter could arise from either incomplete or nonuniform substrate binding. Their absence is a manifestation of the enzyme's high equilibrium association constant for (*cis, syn*)-CPDs in DNA, its ability to discriminate damaged from undamaged DNA (Sancar, 1990; Husain and Sancar, 1987; Li and Sancar, 1990; Baer and Sancar, 1993), and evidence for one specific substrate-enzyme geometry configuration.

The assignment of hyperfine lines as shown in Fig. 2 *A* is following the assignment from previous ENDOR experiments on DNA photolyase (Kay et al., 1999). The hfcs extracted from the ENDOR spectra of the enzyme with and without bound CPD substrate are summarized in Table 1. Hfcs originating from the methyl protons at C(8α) (Fig. 2 *C*) and from the two magnetically inequivalent protons at C(1′) (Fig. 2 *B* and *E*) of the isoalloxazine moiety are more strongly altered compared with the coupling arising from H(6) (Fig. 2 *D*). The latter proton should be further apart from the CPD lesion because this side of the isoalloxazine ring is facing the interior of the enzyme rather than the substrate binding pocket (Park et al., 1995). However, to infer the proximity of a given proton to the CDP lesion from the magnitude of the change of its hfc upon substrate binding could be misleading; the H(6), for example, often seems to be a poor probe for substrate binding in flavoenzymes in general, as its hfc rarely changes when substrates are added (Medina et al., 1994, 1995; Çinkaya et al., 1997). In contrast, the hfc of the C(8α) methyl protons often changes significantly even in cases where the substrate approaches the isoalloxazine from the side opposite to C(8) (Medina et al., 1994).

TABLE 1 Proton hfcs obtained from analysis of the respective EPR and ENDOR spectra with and without bound CPD substrate

Atom position		DNA photolyase without substrate	DNA photolyase with substrate
$CH_3(8\alpha)$	$A_{\parallel}$	8.46	8.39
	$A_{\perp}$	6.99	6.92
	A_{iso}	7.48	7.41
H(6)	A_{iso}	−4.80*	−4.84*
H(5)	A_{iso}	−23*†	−23*†
H(1′) (pro-S)	$A_{\parallel}$	11.33	11.17
H(1′) (pro-R)	$A_{\parallel}$	2.96	2.98
	$A_{\perp}$	2.39	2.33
	A_{iso}	2.58	2.55

All values in MHz; experimental errors ±0.01 MHz, except for the hfc of H(5) with an error of ±1 MHz. For a detailed description of the assignment of the hyperfine lines to the respective proton positions in $FADH^{\bullet}$, see Kay et al. (1999). For the molecular numbering scheme, see Fig. 3. The pro-R and pro-S positions correspond to H^w and H^s in (Kay et al., 1999), respectively. The A_{iso} were calculated from the measured principal values $A_{\parallel}$ and $A_{\perp}$ of the respective hfc tensors: $A_{iso} = (A_{\parallel} + 2A_{\perp})/3$.
*Sign of hfc has not been determined experimentally.
†From EPR spectra.

In ENDOR studies of the isolated photolyase enzyme, the two isotropic hfcs arising from the protons at C(8α) and C(6) were found to be very small compared with the corresponding values from other flavoenzymes (Kay et al., 1999). This finding was attributed to the relatively nonpolar binding site of the FAD in photolyases. Accordingly, the yet smaller hfcs measured for the enzyme bound to the CPD substrate (Table 1) imply an even more nonpolar cofactor environment compared with the flavin binding site of the enzyme alone. Nevertheless, environmental changes may not be the only explanation for altered hfcs in DNA photolyase observed upon substrate binding. Additionally, geometric changes of the flavin cofactor itself should also be considered; using the ratio of the two hfcs of the magnetically inequivalent H(1′) protons, the geometrical arrangement of the ribityl side chain with respect to the isoalloxazine moiety has been determined. This is based on a hyperconjugation model derived for β-protons that are attached to π-electron system (Heller and McConnell, 1960; Horsfield et al., 1961). A value of 170.4 $\pm$ 1° has been found for the dihedral angle between the C(1′)−C(2′) bond and the $2p_z$ orbital at N(10) in the isolated enzyme (Kay et al., 1999). Within an error margin of $\pm$2%, this angle is preserved when using the hfcs determined for the enzyme-substrate complex. Although a preserved arrangement of the ribityl side chain does not exclude the possibility of other geometrical changes occurring within the isoalloxazine ring, such changes, nevertheless, appear rather improbable given the large distance of the isoalloxazine to the CPD lesion.

Model calculations

To date, no crystal structure of a photolyase enzyme with a bound CPD substrate exists. Therefore, it is necessary to indirectly assess the geometrical changes of the redox-active cofactor and its local environment as a result of substrate binding. From a comparison of ENDOR spectra of the enzyme with and without substrate, two main conclusions may be drawn: no major structural alterations of the apoprotein within a 0.6-nm radius surrounding the isoalloxazine moiety of $FADH^{\bullet}$ occur; and the observation of very subtle shifts of the various hfcs implies some environmental changes, which could arise from displacement of water molecules by the substrate in the enzyme's binding pocket. Such changes would locally alter the polarity of the cofactor environment and, therefore, can have an influence on the spin density distribution of $FADH^{\bullet}$ and its isotropic hfcs.

Altered hfcs are frequently observed in spin label EPR when one and the same spin probe is exposed to protein regions or solvents of different polarity (Kawamura et al., 1967; Joela and Lehtovuori, 1999; Steinhoff et al., 2000). Whereas such effects are well documented for nitroxide spin labels, the effect of a modified dielectric environment on flavin proton hfcs has not yet been quantified. Therefore, we have examined solvent effects on spin density distribution and isotropic hfcs by performing model calculations based on DFT at the B3LYP/6–31G* level of theory. The IPCM model (Foresman et al., 1996) has been used to evaluate isotropic proton hfcs of $FADH^{\bullet}$ as a function of the dielectric constant, ϵ_r, of the surrounding continuum. The main purpose of our calculations is to predict trends for solvent-dependent changes of proton hfcs of flavins in general and not to achieve a quantitative agreement with the measured values. The results for the most easily determined proton hfcs are depicted in Fig. 3.

When the polarity of the surrounding medium is increased the positive hfcs of the β-protons at C(8α) and C(1′) are increased compared with their *in vacuo* values at $\epsilon_r = 1$, and the positive spin densities at these positions become enlarged. A similar effect is observed for H(5): the negative isotropic hfc becomes increasingly negative in more polar solvents. Thus, the H(5)'s negative spin density is increased. Contrary to these trends, the spin density at H(6) decreases upon increase of ϵ_r. This is reflected in its decreasing isotropic hfc (becoming less negative) when going to more polar solvents.

Relative to its respective *in vacuo* value at $\epsilon_r = 1$, the isotropic hfc of the pro-R proton (Fig. 3, *solid square*) at C(1′) shows the most pronounced shift when the polarity of the surrounding medium is increased. Somewhat smaller relative changes are calculated for the protons at C(8α) and the pro-S proton (Fig. 3, *open square*) at C(1′). This shows that the isotropic hfcs of these nuclei represent sensitive probes for the local environment of a neutral flavin radical, as has been demonstrated in a variety of ENDOR studies on flavoenzymes (Medina et al., 1994, 1995, 1999; Çinkaya et al., 1997). Although the absolute change of the H(5)'s hfc is largest, its relative change compared with the *in vacuo* value is smaller than those of H(8α) and H(1′). For H(6) only very small relative changes of its hfc are calculated. Hence, this coupling seems to be rather insensitive to changes of solvent polarities, as has been observed in several previous ENDOR studies.

The relative changes of the experimentally determined proton hfcs of the enzyme with bound CPD substrate show a common trend consistent with a change from a more polar to a less polar environment. Also, the amplitudes of the shifts of the proton hfcs seem to be in fair agreement with our model calculations. It follows that the insertion of the substrate into the enzyme's binding pocket renders the apoprotein at the cofactor binding site more nonpolar. This may be explained in terms of a replacement of water molecules as the substrate is inserted into the enzyme's substrate binding pocket. This explanation is also in accordance with the reduced intensity of the matrix ENDOR line observed for the enzyme-substrate complex.

It is unfortunate that the H(5) hfc is not easily detected in cw ENDOR because of its large spectral anisotropy. However, the value obtained from cw EPR is associated with a relatively large experimental error of $\pm$1 MHz (Kay et al.,

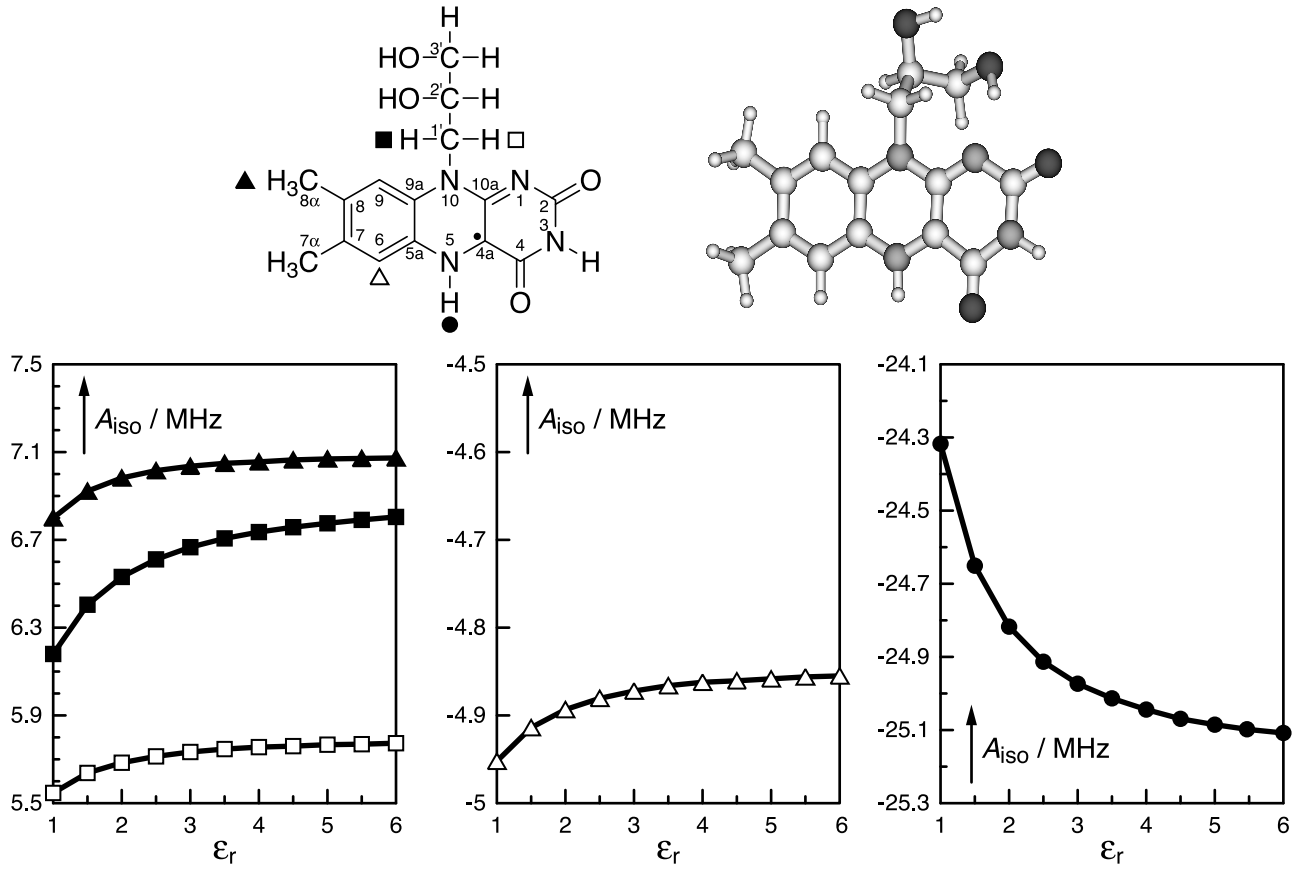

FIGURE 3 Density functional theory calculations of isotropic hfcs of various protons within the 7,8-dimethyl isoalloxazine moiety of an *in vacuo* optimized neutral flavin radical as a function of the dielectric constant ϵ_r. The solvent dependence of the hfcs has been calculated using the IPCM model at the B3LYP/6–31G* level of theory. (*Left panel)* Isotropic hfcs of the protons at C(8α) (*filled triangles*) and C(1′) (*filled* and *open squares*). (*Center panel*) Isotropic hfc of H(6) (*open triangles*). (*Right panel)* Isotropic hfc of H(5) (*filled circles*).

1999). Hence, the subtle shifts predicted for the H(5) coupling when going from a more polar to a less polar cofactor environment may be obscured by the anisotropies introduced by other nuclei, such as the nitrogens. In contrast, the H(5) may not experience the same changes that the other protons do, perhaps, because this proton is hydrogen-bound to the nearby N378 and, therefore, is facing the interior of the enzyme rather than the substrate binding pocket. Nevertheless, additional studies to detect the entire hyperfine tensor of H(5) are indispensable to further substantiate the structural changes introduced upon substrate association. Studies along these lines, such as Davis pulsed ENDOR experiments, are currently in progress and will be published in a subsequent contribution.

CONCLUSIONS

Using EPR and ENDOR spectroscopy we have demonstrated that the geometrical and electronic structure of the FAD cofactor binding site of the DNA photolyase enzyme from *E. coli* is largely conserved when the CPD substrate is bound to the enzyme.

When compared with the ENDOR spectrum of the isolated enzyme, no line-broadening effects are detected in the enzyme-substrate complex, nor are any additional hfcs originating from the CPD lesion in the damaged DNA fragment observed. Based on the similarity of the ENDOR spectra, substantial geometry alterations of the cofactor and its apoprotein site are excluded.

Therefore, we conclude that the CPD segment is placed at least 0.6 nm from the redox-active 7,8-dimethyl isoalloxazine ring of $FADH^{\bullet}$. The results support those model calculations that predict a large distance between these moieties, but oppose models that predict van der Waals contact between the redox partners. To investigate whether the size of the substrate affects its proximity to the cofactor, we therefore plan EPR/ENDOR experiments using model thymine dimers instead of the (physiologically more relevant) UV-irradiated oligo$(dT)_{18}$ DNA substrate examined in the present study.

The observed subtle shifts of the isotropic proton hfcs of the isoalloxazine can be rationalized in terms of a changing polarity of the cofactor environment once the substrate is docked to the enzyme. The trends observed are consistent

with the substrate binding pocket becoming more nonpolar because of the displacement of water molecules upon substrate binding. Model calculations based on density functional theory support the hypothesis that proton hfcs can be sensitive probes for studies of cofactor-protein interactions in flavoenzymes such as photolyases.

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