Affinity Probing of Flavin Binding Sites. 2. Identification of a Reactive Cysteine in the Flavin Domain of *Escherichia coli* DNA Photolyase†

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ABSTRACT: 8-(Methylsulfonyl)FAD reacts with a single cysteine residue (Cys293) in the flavin domain of *Escherichia coli* DNA photolyase to form an 8-(cysteinyl)FAD derivative covalently bound to the protein. About 80% protection against covalent attachment with 8-(methylsulfonyl)FAD was observed in the presence of an equimolar amount of FAD. Flavinylated photolyase retains the ability to repair pyrimidine dimers (15% of native activity) and to bind its antenna chromophore, 5,10-methenyltetrahydrofolate. Comparison of the properties of flavinylated enzyme with photolyase containing noncovalently bound 8-(methylthio)-FAD indicate that a perturbation is necessary to accommodate covalent bond formation. 8-(Methylthio)-FAD-reconstituted enzyme exhibits 95% of native activity. The aerobic stability of fully reduced and radical forms of 8-(methylthio)FAD enzyme is similar to that of native enzyme, whereas a radical form is not detected with flavinylated enzyme and the fully reduced enzyme is more easily oxidized by oxygen. The flavin in 8-(methylthio)FAD enzyme or flavinylated photolyase is shielded from solvent. However, the flavin environment in flavinylated enzyme is less hydrophobic as judged by spectral comparison with model 8-(alkylthio)flavins in various solvents. Enzyme containing noncovalently bound 8-(methylsulfonyl)-FAD was prepared by reconstitution with the fully reduced flavin which does not undergo covalent attachment. Covalent attachment was observed after reoxidation but probably involved dissociation and rebinding of oxidized 8-(methylsulfonyl)FAD. The results show that 8-(cysteinyl)FAD in flavinylated photolyase is at or near the normal flavin binding site. Although Cys293 in the native structure is probably not in the optimal orientation for nucleophilic attack at C(8), the adjustment needed for covalent bond formation is not sufficient to grossly interfere with the enzyme's ability to repair DNA or to interact with its antenna chromophore.

Exposure of DNA to ultraviolet light results in the formation of cyclobutane dimers between two adjacent pyrimidine residues. The damage can be repaired by DNA photolyase in a rather unique enzymatic reaction where visible light acts as a second substrate. Visible light energy is absorbed by one of two known antenna chromophores (5,10-CH^+-H_4folate[1] or a 5-deazaflavin derivative) and then transferred to the reaction center chromophore (fully reduced FAD). The singlet excited state of fully reduced FAD, generated by singlet–singlet interchromophore energy transfer or by direct light absorption, initiates DNA repair by transferring an electron to substrate, forming an unstable pyrimidine dimer radical anion which can rapidly monomerize (Lipman & Jorns, 1992; Ramsey et al., 1992; Jorns et al., 1990; Jorns, 1990; Jordan & Jorns, 1988; Rustandi and Jorns, unpublished results).

Two classes of photolyases can be distinguished on the basis of the nature of the antenna chromophore, whereas all known enzymes contain the same reaction center chromophore. A two-domain structure has been proposed for photolyases. The flavin domain coincides with the more highly conserved C-terminal half of the protein, while the antenna domain is assigned to the N-terminal half where sequence homology is more limited and tends to be restricted to members of the same class (Malhotra et al., 1992; Batschauer, 1993).

With the exception of a recently identified plant blue light photoreceptor (Ahmad & Cashmore, 1993), no sequence homology has been detected between photolyases and other enzymes. The lack of homology with other flavin-containing enzymes is striking and suggests a very special flavin environment in photolyases. This is perhaps not unexpected since photolyases are the only known flavoenzymes that use light as a substrate. Also, the requirement of fully reduced flavin for photolyase catalysis is observed with only one other flavoenzyme, chorismate synthase (Ramjee et al., 1992).

Various low-potential flavin analogues have previously been used in studies with *Escherichia coli* DNA photolyase to probe the flavin environment and mechanism of catalysis (Ramsey & Jorns, 1992; Chanderkar & Jorns, 1991). A high-potential flavin analogue is generated by replacing the methyl group at position C(8) in FAD with a methylsulfonyl ($MeSO_2$) moiety. The 8-$MeSO_2$ substituent undergoes a facile displacement reaction with thiols to yield 8-(alkylthio)flavins, suggesting that 8-$MeSO_2$-substituted flavins might be useful as a chemically reactive probe for cysteine residues in flavin binding sites (Raibekas et al., 1993). In an accompanying paper we show that 8-methylsulfonylFAD (8-$MeSO_2$FAD) reacts with a cysteine residue near the isoalloxazine ring of FAD in pig heart lipoamide dehydrogenase to yield enzyme containing covalently bound 8-(cysteinyl)FAD (Raibekas & Jorns, 1994). In this paper we show that 8-$MeSO_2$FAD reacts with a single cysteine residue in the flavin domain of *E. coli*

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; 8-$MeSO_2$FAD, 8-(methylsulfonyl)FAD; 8-MeSFAD, 8-(methylthio)FAD; ABD-F, 7-fluoro-4-sulfamoyl-2,1,3-benzoxadiazole; TBP, tributylphosphine; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; DTT, dithiothreitol; 5,10-CH^+-H_4folate, 5,10-methenyltetrahydrofolate; SDS, sodium dodecyl sulfate; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide.

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DNA photolyase to yield a flavinylated protein which retains the ability to repair pyrimidine dimers and to bind its antenna chromophore, 5,10-CH^+-H_4folate.

EXPERIMENTAL PROCEDURES

Materials. Modified trypsin was from Promega. Unmodified trypsin was from Boehringer. Protein standards used for SDS–PAGE electrophoresis, except for myoglobin fragments, were from Bio-Rad. Thermolysin, myoglobin fragments, 5-formyltetrahydrofolate, phosphodiesterase (*Naja naja* snake venom), and alkaline phosphatase were from Sigma. 7-Fluoro-4-sulfamoyl-2,1,3-benzoxadiazole (ABD-F) was obtained from Wako Chemicals. Tributylphosphine (TBP) was from Aldrich. Phenyl-Sepharose CL-4B was purchased from Pharmacia. Guanidine hydrochloride was from Heico. Oligo-$(dT)_{18}$ was obtained from University of Pennsylvania DNA Synthesis Service. 8-Chlororiboflavin was a generous gift from Dr. John P. Lambooy.

Chromatography and Spectroscopy. Analysis of proteolytic fragments and purification of target peptides were performed, in part, using a Rainin gradient HPLC system equipped with a Vydac 218TP54 (C_{18}, 5-μm, 46 × 250 mm) reversed-phase column (The Separation Group) or a PolySULFOETHYL Aspartamide SCX (46 × 200 mm) HPLC column (The Nest Group). Absorption spectra were recorded using a Perkin-Elmer Lambda 3B or Lambda 2S spectrophotometer.

Amino Acid Sequence Analysis. Purified peptides (50–100 pmol) were sequenced by Edman degradation on an Applied Biosystems 477A protein sequencer equipped with an Applied Biosystems 120A Analyzer at the Laboratory for Macromolecular Analysis at the Albert Einstein College of Medicine, Bronx, NY.

Preparation of Native Enzyme and Apophotolyase. DNA photolyase from *E. coli* was purified as previously described (Jorns et al., 1987). The concentration of native photolyase was calculated on the basis of the FAD radical absorbance at 580 nm (ϵ_{580} = 4800 M^{-1} cm^{-1}) (Wang & Jorns, 1989). The apoenzyme was prepared by the phenyl-Sepharose column chromatography method described by Jorns et al. (1990) except that DTT was omitted from elution buffers. The concentration of apophotolyase (ϵ_{280} = 100 100 M^{-1} cm^{-1}) was calculated on the basis of protein absorbance at 280 nm (Wang & Jorns, 1989). The apoenzyme was stored at −20 °C in 0.1 M potassium phosphate buffer, pH 7.0, containing 50% ethylene glycol and 0.5 mM EDTA (buffer A).

Catalytic Assays. Photolyase assays were performed at 21 °C with UV-irradiated oligo$(dT)_{18}$ as substrate, similar to those described by Jorns et al. (1985). Anaerobic and aerobic assays were initiated by addition of enzyme to buffer [50 mM Tris-HCl, pH 7.2, containing 10 mM NaCl, 1 mM EDTA, and UV-irradiated oligo$(dT)_{18}$ (5.4 μM with respect to dimer, unless otherwise stated)] containing 0 or 1 mM DTT, respectively. UV-irradiated oligo$(dT)_{18}$ was prepared as previously described (Jorns et al., 1985). Enzyme concentration was estimated on the basis of flavin content.

Reconstitution of Apophotolyase with Oxidized 8-(Methylsulfonyl)FAD. 8-$MeSO_2$FAD was prepared as described by Raibekas et al. (1993). Apoenzyme (10^{-5} M) in buffer A was incubated with a 4–5-fold excess of oxidized 8-$MeSO_2$-FAD for 23 h at 4 °C. Unbound flavin was removed and the buffer was changed to 0.1 M potassium phosphate, pH 7.0, containing 0.5 mM EDTA and 20% glycerol (storage buffer), by using a Centricon-10 and/or Microcon-10 microconcentrator. The reconstituted enzyme was stored at −20 °C.

As will be described, enzyme reconstituted with oxidized 8-$MeSO_2$FAD contains covalently bound flavin [8-(cysteinyl)-FAD]. To determine the covalent flavin content in these flavinylated enzyme preparations, protein concentration was determined on the basis of the absorbance of the intact enzyme at 280 nm after correcting for the flavin contribution. Flavin was determined using the extinction coefficient determined for the enzyme at 464 nm (ϵ_{464} = 20 400 M^{-1} cm^{-1}). The flavin contribution at 280 nm was estimated on the basis of the absorbance of free 8-(*N*-acetylcysteinyl)FAD (ϵ_{280} = 18 900 M^{-1} cm^{-1}) (Raibekas & Jorns, 1994). Alternatively, the reconstituted enzyme was precipitated by addition of 5% TCA. The protein pellet was redissolved in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 5 M guanidine hydrochloride. Protein was estimated as above. Flavin was determined using the extinction coefficient reported for free 8-(*N*-acetylcysteinyl)FAD (ϵ_{480} = 25 200 M^{-1} cm^{-1}) (Moore et al., 1978). Similar results were obtained by either method.

Preparation and Electrophoresis of Tryptic Digests of Flavinylated Enzyme. Flavinylated enzyme (prepared by reconstitution with oxidized 8-$MeSO_2$FAD) and apoenzyme control samples were labeled with ABD-F as described in the next section. The ABD-F-labeled preparations were digested with unmodified trypsin, and the tryptic digests were then analyzed by SDS–PAGE. Digestions were conducted at an enzyme to substrate ratio of 1:20 in 0.05 M Tris-HCl, pH 8.5, containing 0.05% SDS and 20% DMF. An additional aliquot of trypsin was added after 3 h at 37 °C. Aliquots were withdrawn at 3 and 6 h, diluted 1:1 with 0.2 M Tris-HCl buffer, pH 6.8, containing 45% glycerol, 6% SDS, 3% β-mercaptoethanol, and 0.005% bromphenol blue, and stored at −20 °C until all samples were collected. SDS–PAGE was performed as described by Schagger and von Jagow (1987). Proteins were stained with Coomassie blue. The following proteins were used as standards: rabbit muscle phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and various myoglobin fragments (14.5, 10.6 and 6.2 kDa).

Isolation of 8-(Cysteinyl)FAD-Containing Peptides from Flavinylated Enzyme. Prior to alkylation of cysteine residues, the storage buffer for flavinylated enzyme (prepared by reconstitution with oxidized 8-$MeSO_2$FAD) was changed to ABD-F buffer (0.1 M sodium borate, pH 8.0, containing 2 mM EDTA and 0.5% SDS) using a Microcon-10 microconcentrator. Reduction and alkylation of sulfhydryl groups were conducted by incubating the flavinylated enzyme in ABD-F buffer containing 2 mM TBP and 4 mM ABD-F for 1 h at 37 °C, following the procedure described by Kirley (1989). Excess reagents were removed and the buffer was changed to a digestion buffer using a microconcentrator. The sample stored at 4 °C until use.

Proteolytic digestion was conducted in 0.1 M Tris-HCl buffer, pH 8.0, containing 2 mM calcium chloride and 2 M urea at 37 °C. Digestion was initiated using modified trypsin at an enzyme to substrate ratio of 1:5 (w/w). After 4.5 h, thermolysin was added at an enzyme to substrate ratio of 1:10, and the incubation was continued for an additional 18 h. The digest was applied to a Vydac C_{18} reversed-phase HPLC column. Elution was conducted using a linear gradient of acetonitrile (5–40%, unless otherwise noted) in the presence of 0.1% TFA. The gradient was developed over 34 min at a flow rate of 1 mL/min. Two 8-(cysteinyl)FAD-containing peptide fractions were identified by their absorbance at 480

nm, dried *in vacuo*, and redissolved in a minimal volume (30–50 μL) of 10 mM ammonium acetate buffer, pH 4.5, containing 10% acetonitrile. The samples were applied separately to a PolySULFOETHYL Aspartamide SCX HPLC column and eluted with a linear gradient of potassium chloride (0–0.037 M) in 5 mM potassium phosphate buffer, pH 3.0, containing 10% acetonitrile. The gradient was developed over 32 min at a flow rate of 1 mL/min. For desalting, the two purified 8-(cysteinyl)FAD-containing peptides were reapplied to a Vydac C_{18} reversed-phase column, eluted with a gradient of acetonitrile (5–75%) in 0.1% TFA, dried *in vacuo*, and then stored at −20 °C.

Binding of 5,10-CH$^+$-H$_4$folate to Flavinylated Enzyme. Flavinylated enzyme was prepared by reconstitution with oxidized 8-MeSO_2FAD. To determine whether the pterin binding site was affected by the introduction of a covalent flavin linkage, flavinylated enzyme (1 mL, 10^{-5} M protein) was incubated with a 50-fold excess of 5,10-CH$^+$-H$_4$folate at 3 °C in storage buffer containing 10 mM DTT to protect against pterin oxidation. After 13.5 h, another aliquot of 5,10-CH$^+$-H$_4$folate was added and the incubation continued for an additional 9 h. Solid ammonium sulfate was added to a concentration of 1.7 M, and the sample was applied to a phenyl-Sepharose column (1 mL) equilibrated with storage buffer containing 1.7 M ammonium sulfate. Unbound 5,10-CH$^+$-H$_4$folate was removed by washing the column with equilibration buffer. The enzyme was eluted with buffer A as a sharp band in a total volume of 0.4 mL. The extent of pterin incorporation was estimated on the basis of the absorbance of the bound 5,10-CH$^+$-H$_4$folate at 370 nm using the extinction coefficient reported for the free chromophore (25.1×10^3 M^{-1} cm^{-1}) (Rabinowitz, 1963). 5,10-CH$^+$-H$_4$folate was prepared as described by Rabinowitz (1963).

Reconstitution of Apophotolyase with Reduced 8-(Methylsulfonyl)FAD. 8-MeSO_2FAD was reduced with a 100-fold excess of NADH prior to mixing with an anaerobic solution containing apoenzyme. After incubation for 19–21 h at 4 °C, unbound flavin was removed by passing the enzyme through a Sephadex G-25 column equilibrated with aerobic buffer (0.1 M potassium phosphate, pH 7.0, containing 20% glycerol plus 0.5 mM EDTA). The peak column fraction was used directly in spectral studies to monitor the reoxidation of enzyme-bound reduced 8-MeSO_2FAD. The slower covalent attachment of reoxidized 8-MeSO_2FAD was monitored after concentrating the pooled gel filtration eluate using a Centricon-10 microconcentrator. The concentration of enzyme-bound 8-MeSO_2FAD after reoxidation was estimated using the extinction coefficient of the free flavin ($\epsilon_{450} = 11.2 \times 10^3$ M^{-1} cm^{-1}) (Raibekas et al., 1993). In one experiment, a 5-fold excess of FAD was added to determine whether it might protect against covalent attachment of reoxidized 8-MeSO_2FAD. After incubation for 116 h at 5 °C, samples were precipitated with 5% TCA, and the protein pellet was washed with 5% TCA. To determine the extent of covalent flavin incorporation, the pellet was redissolved in 0.1 M potassium phosphate buffer, pH 7.0, containing 5 M guanidine hydrochloride and 0.5 mM EDTA.

Reconstitution of Apophotolyase with 8-(Methylthio)FAD. 8-Chlororiboflavin was converted to 8-chloroFAD with partially purified flavokinase/FAD synthase from *Brevibacterium ammoniagenes* (Hausinger et al., 1986). 8-(Methylthio)FAD (8-MeSFAD) was prepared using a procedure similar to that described for the riboflavin analogue (Raibekas et al., 1993; Moore et al., 1979). Briefly, 8-chloroFAD (10^{-4} M) was incubated with 16.7 mM sodium thiomethoxide in 50 mM ammonium bicarbonate, pH 8.9 (prebubbled with argon), for 1 h at room temperature and then overnight at 4 °C. The sample was dried under vacuum and stored at −20 °C.

Apophotolyase (10^{-5} M) in buffer A was incubated with a 5-fold excess of oxidized 8-MeSFAD at 4 °C for 23 h. The reconstituted enzyme was isolated by using a Sephadex G-25 gel column equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 20% glycerol (G-25 column buffer). The column eluate was concentrated using an Amicon ultrafiltration cell (PM-10 membrane). The glycerol concentration was adjusted to 50%, and the sample was stored at −20 °C.

The extent of 8-MeSFAD incorporation was determined after denaturation of the reconstituted enzyme in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA, 25% glycerol, and 4 M guanidine hydrochloride. Flavin content and contribution to absorbance at 280 nm were estimated using extinction coefficients determined for free 8-MeSFAD ($\epsilon_{280} = 25.2 \times 10^3$ M^{-1} cm^{-1}, $\epsilon_{483} = 29.9 \times 10^3$ M^{-1} cm^{-1}) in a similar buffer (50 mM potassium phosphate pH 7.0 containing 75 mM sodium chloride, 25 mM EDTA, 25% glycerol, and 4 M guanidine hydrochloride). The absorption maximum of free 8-MeSFAD shifts from 483 to 479 nm ($\epsilon_{479} = 27.4 \times 10^3$ M^{-1} cm^{-1}) when glycerol and guanidine hydrochloride are omitted from the buffer.

Extinction coefficients for 8-MeSFAD were first determined in the absence of glycerol and guanidine hydrochloride and then corrected for the perturbation observed in the presence of these reagents. 8-MeSFAD was converted to 8-(methylthio)riboflavin ($\lambda_{max} = 476$ in 0.1 M potassium phosphate pH 7.0 or 0.05 M ammonium bicarbonate, pH 9.0) by incubation for 1 h with phosphodiesterase (*Naja naja* snake venom) and alkaline phosphatase under conditions described by Raibekas et al. (1993). 8-(Methylthio)riboflavin was estimated using a previously reported extinction coefficient ($\epsilon_{474} = 28\,500$ M^{-1} cm^{-1} in 0.1 M potassium phosphate pH 7.0) (Moore et al., 1979).

Preparation of 8-(Methylthio)FAD Radical Bound to Photolyase. Enzyme reconstituted with oxidized 8-MeSFAD was fully reduced by reaction with 5 mM sodium dithionite in aerobic 0.1 M potassium phosphate buffer, pH 7.0, containing 0.75 mM EDTA and 25% glycerol. Excess dithionite was removed and the buffer was changed to G-25 column buffer using an Amicon ultrafiltration cell (three cycles). The glycerol concentration was then adjusted to 50%. The flavin content of the preparation was estimated after denaturation with guanidine hydrochloride as described in the preceding section.

Effect of Substrate on the Aerobic Stability of Fully Reduced 8-(Methylthio)FAD Bound to Photolyase. Enzyme containing 8-MeSFAD radical was photoreduced under anaerobic conditions with blue/black or red light in the absence or presence of UV-irradiated oligo(dT)$_{18}$ (2.3 mol of dimer/mol of flavin), respectively). Reactions were conducted in 50 mM Tris-HCl buffer, pH 7.2, containing 17 mM NaCl, 1 mM EDTA, 2 mM DTT, and 9% glycerol. Fully reduced enzyme samples were made aerobic, and reoxidation to the radical form was monitored at 8 °C.

RESULTS

Reconstitution of Apophotolyase with Oxidized 8-(Methylsulfonyl)FAD. Apophotolyase was prepared by a phenyl-Sepharose column chromatography procedure (Jorns et al., 1990). The absorption spectrum of enzyme reconstituted with oxidized 8-MeSO_2FAD exhibits a single, very broad band in

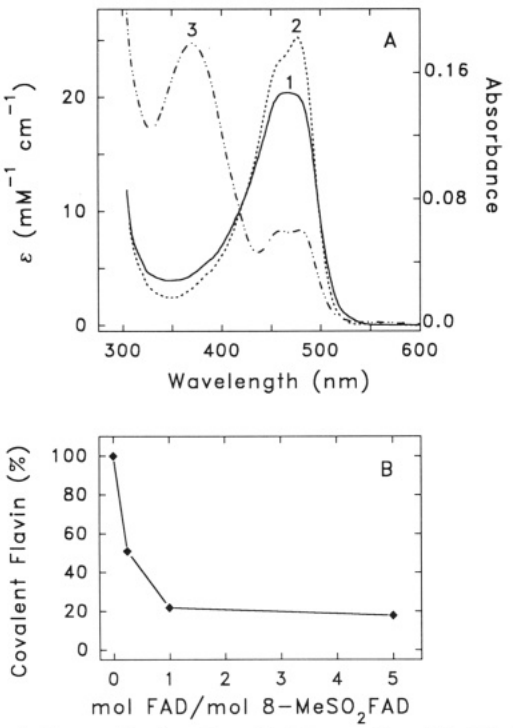


FIGURE 1: Reconstitution of apophotolyase with oxidized 8-(methylsulfonyl)FAD. (Panel A) Spectral properties of the reconstituted enzyme. Curve 1 shows the absorption spectrum of a dilution of reconstituted enzyme in 0.1 M potassium phosphate buffer, pH 7.0. Curve 2 was recorded for a duplicate sample prepared in the same buffer containing 6.0 M guanidine hydrochloride. Curves 1 and 2 are plotted according to the left-hand axis. In a separate experiment, flavinylated enzyme (isolated after reconstitution with oxidized 8-$MeSO_2FAD$) was incubated with 5,10-CH^+-H_4folate and then reisolated as described under Experimental Procedures. Curve 3 is the spectrum recorded for the reisolated enzyme in buffer A, plotted according to the right-hand axis. (Panel B) Protection against covalent flavin incorporation in the presence of FAD. Reaction mixtures containing apophotolyase (9 μM) in buffer A were incubated at 4 °C in the presence of 40 μM 8-$MeSO_2FAD$ plus a variable amount of FAD. After 11 h, the protein was precipitated by addition of 5% TCA, and the extent of covalent flavin incorporation was determined as described under Experimental Procedures. Values are expressed relative to a control sample, incubated in the absence of FAD, which contained 0.81 mol of flavin per mol of protein.

the visible region with a maximum at 464 nm (Figure 1A, curve 1). The spectrum observed after denaturation with guanidine hydrochloride (λ_{max} = 478 nm) (Figure 1A, curve 2) is similar to that observed for free 8-(cysteinyl)FAD in water (λ_{max} = 480 nm) (Moore et al., 1978) and quite distinct as compared with free 8-$MeSO_2FAD$ (λ_{max} = 450, 333 nm) (Raibekas et al., 1993).

When the reconstituted enzyme was denatured with 5% TCA, all of the flavin could be recovered by redissolving the protein pellet in 6 M guanidine hydrochloride (data not shown). The results suggest that 8-$MeSO_2FAD$ reacts with a cysteine residue in photolyase to form a covalently bound 8-(cysteinyl)-FAD derivative (ϵ_{464} = 20 400 M^{-1} cm^{-1}), similar to that observed with lipoamide dehydrogenase (Raibekas & Jorns, 1994).

The covalently attached flavin in reconstituted enzyme (0.8 mol of flavin per mol of protein) appears to be shielded from solvent, as judged by comparison of its spectral properties with 8-(methylthio)riboflavin in various water/DMSO mixtures [see Raibekas and Jorns (1994) and the inset in Figure 7]. Normal FAD bound to photolyase is also shielded from solvent and exhibits spectral properties similar to those of the corresponding free flavin in nonpolar solvents (Jorns et al., 1990).

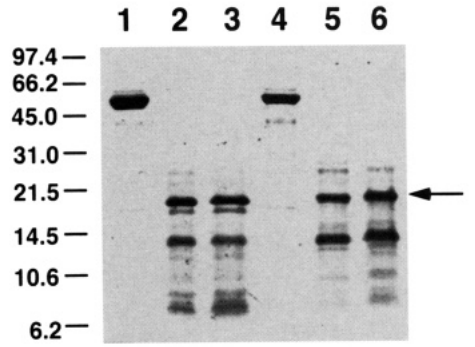


FIGURE 2: SDS–PAGE analysis of tryptic digests obtained with 8-(methylsulfonyl)FAD-reconstituted photolyase (flavinylated enzyme) or apophotolyase. Photolyase samples were labeled with ABD-F and then digested with unmodified trypsin. Lanes 1 and 4 were obtained for undigested ABD-F-labeled apophotolyase and flavinylated enzyme, respectively. Aliquots of ABD-F-labeled apophotolyase subjected to 3 and 6 h of digestion were run in lanes 2 and 3, respectively. Lanes 5 and 6 were obtained for aliquots of ABD-F-labeled flavinylated enzyme after 3 and 6 h of digestion, respectively. The proteins used as molecular weight standards and other experimental details are described under Experimental Procedures.

Covalent Flavinylation of Photolyase in the Presence of FAD. The extent of covalent flavinylation of photolyase was decreased when reconstitution reactions with 8-$MeSO_2FAD$ were conducted in the presence of FAD. About 80% protection was observed with equimolar concentrations of FAD and 8-$MeSO_2FAD$ (Figure 1B), similar to that observed with lipoamide dehydrogenase (Raibekas & Jorns, 1994). The results suggest that 8-$MeSO_2FAD$ reacts with a cysteine residue at or near the photolyase active site.

Identification of the Covalent Flavin Attachment Site in Photolyase. Photolyase samples were subjected to alkylation with ABD-F under conditions (see experimental procedures) where unmodified cysteine residues typically undergo quantitative conversion to a green fluorescent derivative. ABD-F is not expected to react with the putative, flavinylated cysteine (nonfluorescent) in enzyme reconstituted with oxidized 8-$MeSO_2FAD$. After being labeled with ABD-F, photolyase samples were digested with trypsin, and reaction progress was monitored by SDS–PAGE. Two major peptides (20.4 and 13.5 kDa) were observed after digestion of reconstituted enzyme for 3 or 6 h (Figure 2, lane 5 and 6, respectively). Essentially similar results were obtained with apoenzyme, except that the 13.5-kDa band was somewhat less prominent (Figure 2, lanes 2 and 3, respectively). The 20.4- and 13.5-kDa peptides in the apoenzyme digest and the 13.5-kDa peptide in the reconstituted enzyme digest exhibited green fluorescence when examined under UV light prior to staining for protein. The 20.4-kDa band in the reconstituted enzyme digest was nonfluorescent but exhibited a yellow color under white light, suggesting that the peptide contained a flavinylated cysteine residue.

E. coli photolyase contains seven cysteine residues, clustered mainly in the amino-terminal half of the protein. A tryptic peptide as large as 20.4-kDa with only one cysteine is possible only for the cysteine (Cys293) closest to the carboxyl terminus in the intact enzyme. Tryptic peptides containing only Cys293 could range in size from 18.4 to 25.5 kDa. The largest peptide containing only one of the other cysteine residues could not exceed 11.2 kDa (Figure 3).

To obtain direct evidence that Cys293 is the flavinylation site, shorter peptides suitable for amino acid sequence analysis were sought by digesting ABD-F-labeled reconstituted enzyme with modified trypsin followed by digestion with thermolysin. The digest was chromatographed on a Vydac C_{18} HPLC

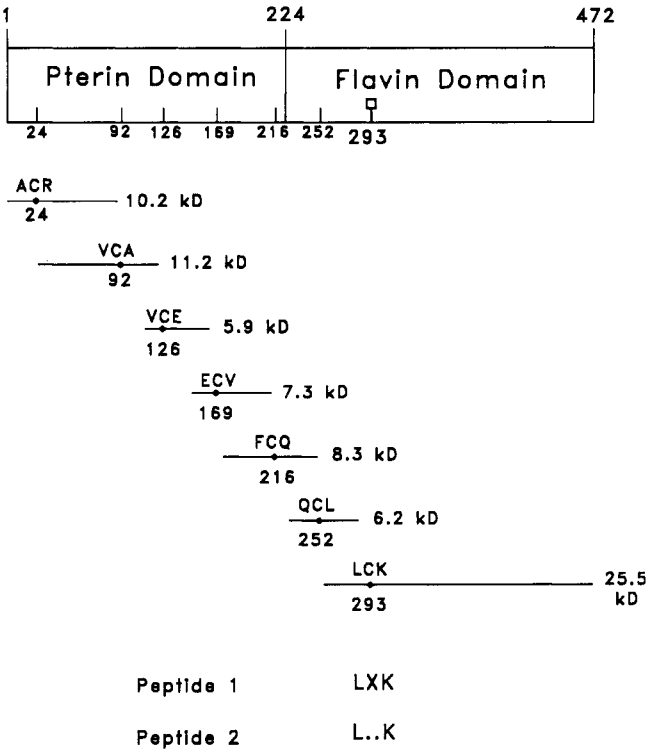


FIGURE 3: Identification of the 8-(cysteinyl)FAD covalent attachment site in flavinylated photolyase. The top panel shows the domain structure of *E. coli* photolyase as deduced by comparison with results obtained with the homologous yeast photolyase in limited proteolytic digestion studies (Malhotra et al., 1992). The middle portion of the figure shows the seven largest peptides, each containing a single cysteine residue, which could be generated by tryptic digestion of *E. coli* photolyase. The amino acids adjacent to each cysteine are shown for comparison with the sequence determined for the two flavinylated tripeptides. [Amino acid numbering starts with the initial methionine and differs by one unit as compared with the reported protein sequence (Sancar et al., 1984) where numbering was started from the second amino acid.] An unknown amino acid derivative, X, was detected in the second cycle in the sequencing of peptide 1. No amino acid derivative was detected at this position in peptide 2.

column and elution profiles were obtained by monitoring absorbance at 220 and 480 nm in duplicate analytical runs (Figure 4). Two flavin-containing peptides were detected, as judged by the 480-nm elution profile where most of the 480-nm absorbance eluted with the faster moving band (peptide 1). Peptides 1 and 2 were isolated in a preparative scale run and then separately purified on a PolySULFOETHYL Aspartamide SCX HPLC column where most (85%) of the 480-nm absorbance eluted in a single peak (data not shown).

Amino acid sequence analysis showed that peptides 1 and 2 were both tripeptides with identical N- and C-terminal residues. An unknown amino acid, X, was detected during the second cycle of the analysis with peptide 1 (LeuXLys) whereas no signal was detected in the second cycle with peptide 2 (Leu···Lys). Comparison of the amino acid sequence data obtained for peptides 1 and 2 with the amino acids adjacent to each of the seven cysteines in photolyase unambiguously identifies Cys293 as the flavin attachment site (Figure 3). The basis for the different chromatography and sequence analysis behavior observed with the two photolyase peptides is not clear but may perhaps result from some modification of the flavinylated cysteine during proteolytic digestion. The behavior of photolyase peptide 2 during sequence analysis is similar to that observed for the peptide obtained in similar experiments with lipoamide dehydrogenase (Raibekas & Jorns, 1994).

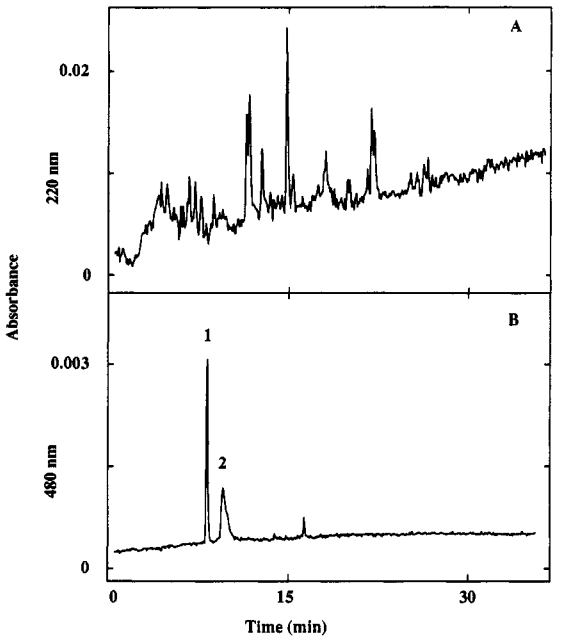


FIGURE 4: Reversed-phase chromatography of a proteolytic digest of flavinylated photolyase. Flavinylated enzyme was prepared by reconstitution with oxidized 8-$MeSO_2$FAD. The preparation was then alkylated with ABD-F, digested with modified trypsin and thermolysin, loaded onto a Vydac C_{18} reversed-phase column, and eluted with a gradient of acetonitrile (5–35%) in the presence of 0.1% TFA. Elution profiles obtained by monitoring absorbance at 214 and 480 nm are shown in panels A and B, respectively.

Does Covalent Flavinylation of Photolyase Interfere with Pterin Binding? Studies by Jorns et al. (1990) show that reconstituted photolyase preparations containing only FAD or pterin (5,10-CH^+-H_4folate) retain the ability to bind the missing chromophore. Flavinylated enzyme might be unable to bind pterin if there was a substantial difference in the location and/or orientation of the covalent flavin as compared with normal, noncovalent FAD.

To test this hypothesis, a sample of flavinylated enzyme [0.8 mol of 8-(cysteinyl)FAD/mol of protein] was incubated with 5,10-CH^+-H_4folate and then adsorbed onto a phenyl-Sepharose column. After being washed to remove unbound 5,10-CH^+-H_4folate, photolyase was eluted as a sharp, brightly fluorescent (blue-green) band. The fluorescence is attributed to bound pterin since similar fluorescence is observed for pterin in native photolyase whereas 8-(cysteinyl)FAD in reconstituted enzyme is nonfluorescent. Pterin incorporation is also accompanied by the appearance of a new band in the absorption spectrum of flavinylated enzyme at 370 nm (Figure 1A, curve 3).

The absorption maximum of free 5,10-CH^+-H_4folate shifts from 360 to 385 nm with hardly any change in extinction coefficient upon binding to apophotolyase or reconstituted enzyme containing noncovalently bound FAD (Jorns et al., 1990). The results show that flavinylation affects the absorption maximum of the bound pterin (370 versus 385 nm). However, the extent of pterin incorporation observed with flavinylated enzyme (0.8 mol of 5,10-CH^+-H_4folate per mol of protein) is similar to that obtained in previous studies with unmodified enzyme. Half of the covalent flavin was lost during reconstitution with 5,10-CH^+-H_4folate, probably via a thiol interchange reaction with DTT (10 mM) added to protect against pterin oxidation. The results show that flavinylation does not prevent pterin binding but does perturb the pterin environment.

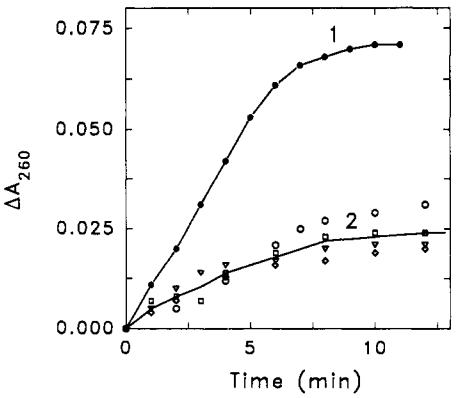


FIGURE 5: Catalytic assays with native photolyase and enzyme reconstituted with oxidized 8-(methylsulfonyl)FAD (flavinylated enzyme). The increase in absorbance at 260 nm, due to repair of dimers in UV-irradiated oligo(dT)$_{18}$ (4 μM with respect to dimer), is plotted versus the time of irradiation with photoreactivating light. Assays were conducted under anaerobic conditions with dithionite-reduced native (6.4×10^{-8} M) or flavinylated enzyme (1.43×10^{-7} M). The data points (filled circles) from a single assay with native enzyme are shown in line 1. Line 2 is the average of four separate assays with flavinylated enzyme (open symbols).

Can Photolyase Containing Covalently Bound 8-(Cysteinyl)FAD Repair Pyrimidine Dimers? Although fully reduced FAD is required for dimer repair, native photolyase or enzyme reconstituted with reduced FAD is isolated with the flavin present as blue, neutral radical. The radical form can be activated via an *in situ* photoreduction when assays are conducted in the presence of DTT or by prior reduction with dithionite (Jorns et al., 1984, 1990). Photolyase containing covalently bound 8-(cysteinyl)FAD is readily reducible by dithionite. Since the reduced flavin is immediately converted to the fully oxidized state upon exposure to air, dimer repair was measured under anaerobic conditions.

Assay mixtures containing enzyme plus UV-irradiated oligo(dT)$_{18}$ were exposed to photoreactivating light, and dimer repair was monitored by the increase in absorbance at 260 nm. Curve 1 in Figure 5 shows the results of a typical assay with dithionite-reduced native enzyme. The rate of dimer repair is linear with respect to the light dose until the reaction is nearly complete, a feature attributable to the low K_m value with this substrate (Jorns et al., 1985). Dimer repair is observed with dithionite-reduced flavinylated enzyme, but the repair rate curves off before all of the dimer is repaired, suggesting a higher K_m value. The activity of the reduced flavinylated enzyme, estimated from initial rate data, was 15% of that observed for native enzyme. No dimer repair was observed with oxidized flavinylated enzyme or upon addition of dithionite alone to the assay mixture.

Reconstitution of Apophotolyase with Reduced 8-(Methylsulfonyl)FAD. Only noncovalent binding can occur upon reconstitution with reduced 8-MeSO$_2$FAD because the oxidized flavin is required for reaction with thiols (Raibekas et al., 1993). However, covalent attachment might occur upon flavin reoxidation, provided that Cys293 is suitably positioned for nucleophilic attack. To test this hypothesis, apophotolyase was reconstituted with reduced 8-MeSO$_2$FAD under anaerobic conditions and then separated from unbound flavin by aerobic gel filtration chromatography. The reduced flavin was bound to the enzyme and remained largely reduced during the chromatography step as judged by the absorption spectrum recorded for the peak protein fraction immediately after isolation (Figure 6A, curve 1). The sample exhibited a peak at 375 nm and a shoulder around 450 nm, similar to that observed for free (λ_{max} = 362, 310 nm) or flavodoxin-bound (λ_{max} = 382, 323 nm) reduced 8-MeSO$_2$flavin (Raibekas et al., 1993; Moore et al., 1979), except that the near UV peak around 320 nm appears to be obscured by the protein end absorbance in photolyase.

Unlike the free flavin, reduced 8-MeSO$_2$FAD bound to photolyase is moderately stable under aerobic conditions. Slow reoxidation occurred over a period of hours at 8 °C, as judged by the increase in absorption at 450 nm, accompanied by the loss of the 375-nm band of reduced enzyme (Figure 6A, curves 2–6). The neutral radical form of 8-MeSO$_2$FAD, known to absorb in the 600 nm region (Raibekas et al., 1993), was not detected during reoxidation.

Dimer repair was observed when enzyme reconstituted with reduced 8-MeSO$_2$FAD was assayed under aerobic conditions. The rate observed immediately after isolation was 6.5% of that observed with dithionite-reduced native enzyme. The reduced flavin was required for activity since no dimer repair was detectable after enzyme reoxidation.

In a separate experiment, enzyme reconstituted with reduced 8-MeSO$_2$FAD was concentrated after gel filtration and then incubated at 5 °C. Curve 1 in Figure 6B shows the absorption spectrum of the reoxidized enzyme, recorded 4 h after isolation (λ_{max} = 450, shoulder at 325 nm). [The near UV absorption band of the oxidized flavin appears to be hypsochromically shifted as compared with free 8-MeSO$_2$FAD (λ_{max} = 450, 333 nm) and partially obscured by the protein end absorption.] Further incubation of the reoxidized enzyme resulted in the covalent attachment of the flavin, as judged by the appearance of an intense, broad absorption band at 464 nm (Figure 6B, curves 2–5). This reaction was quite slow and probably not complete even after 5 days of incubation (see inset to Figure 6B). The spectrum recorded after 5 days (Figure 6B, curve 5) is similar to that observed for enzyme directly reconstituted with oxidized 8-MeSO$_2$FAD (Figure 1A, curve 1), but the extent of covalent flavin incorporation was somewhat lower (0.6 mol versus 0.8 mol of 8-(cysteinyl)FAD/mol of protein).

The rate of covalent flavinylation of apoenzyme with oxidized 8-MeSO$_2$FAD has not been examined in detail but is at least 80% complete after a 23 h incubation at 4 °C. The much slower flavinylation observed after oxidation of enzyme reconstituted with reduced 8-MeSO$_2$FAD suggests that this reaction may involve a conformational change and/or dissociation and rebinding of the reoxidized 8-MeSO$_2$FAD. The latter hypothesis was tested by incubating the reoxidized enzyme with a 5-fold excess of FAD. The extent of covalent flavinylation was decreased by 50%, suggesting that the reaction does involve dissociation/rebinding of 8-MeSO$_2$FAD.

Reconstitution of Apophotolyase with 8-(Methylthio)FAD. The noncovalently bound FAD in native photolyase is replaced with a covalently attached 8-(alkylthio)FAD derivative in flavinylated enzyme. Studies with enzyme containing 8-(methylthio)FAD (8-MeSFAD) were conducted to determine the effect of introducing an alkylthio substituent at position 8 without covalent attachment of the flavin.

Photolyase reconstituted with 8-MeSFAD (0.78 mol of flavin/mol of protein) exhibits two fairly sharp peaks at 482 and 454 nm (Figure 7A, curve 1). Upon denaturation with guanidine hydrochloride (Figure 7A, curve 2), the peak at 482 nm broadens, decreases in intensity, and shifts about 1 nm to longer wavelengths while the peak at 454 nm is replaced by a shoulder around 457 nm. The sharp peaks seen with photolyase-bound 8-MeSFAD are quite different from the single broad peak observed with flavinylated enzyme (see Figure 1, curve 1). Also, the extinction coefficient of

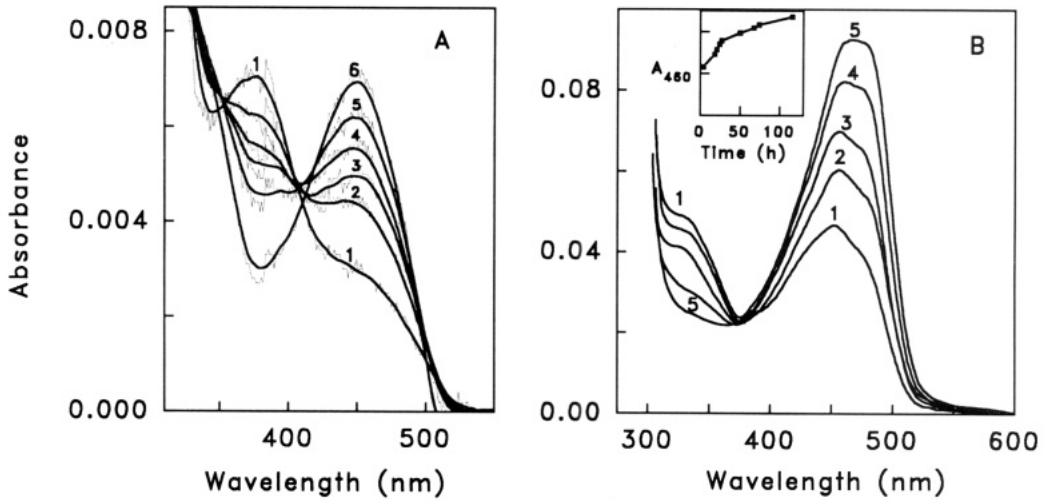


FIGURE 6: Reoxidation of photolyase reconstituted with reduced 8-(methylsulfonyl)FAD and covalent attachment of the reoxidized flavin. Reconstituted enzyme, prepared under anaerobic conditions, was separated from unbound flavin by aerobic gel filtration chromatography. (Panel A) The reoxidation reaction was monitored using the peak protein column fraction. Curve 1 was recorded immediately after isolation. Curves 2–6 were recorded after 60, 100, 135, 195, and 375 min, respectively, incubation at 8 °C in 0.1 potassium phosphate, pH 7.0, containing 20% glycerol and 0.5 mM EDTA. The smoothed curves (solid lines) were generated from the actual data points (dotted lines) using an unweighted running average procedure. (Panel B) The slower covalent attachment of the reoxidized flavin was monitored in a separate experiment after concentrating the pooled gel filtration column protein eluate using a microconcentrator (110 min at 5 °C) followed by incubation at 5 °C. Curves 1–5 were recorded 4, 18.2, 26.8, 67.3, and 115.8 h after elution from the gel filtration column. The inset shows a plot of the increase in absorbance at 460 versus time.

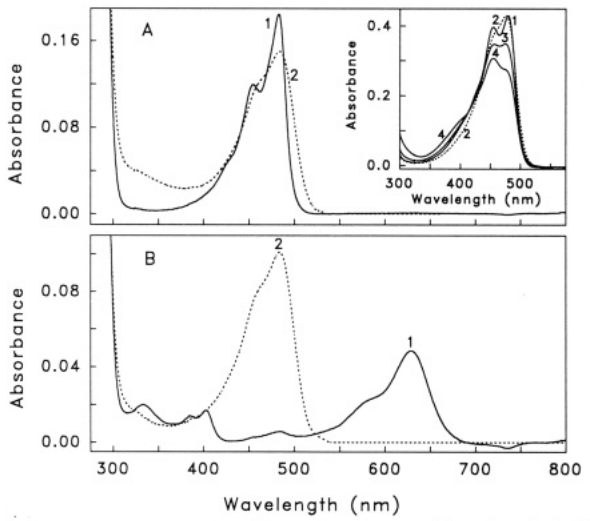


FIGURE 7: Reconstitution of apophotolyase with 8-(methylthio)-FAD. (Panel A) Curve 1 is the absorption spectrum of oxidized 8-MeSFAD bound to photolyase in 0.1 M potassium phosphate, pH 7.0, containing 1 mM EDTA and 50% glycerol. Curve 2 was recorded after denaturation in 0.1 M potassium phosphate, pH 7.0, containing 0.75 mM EDTA, 25% glycerol, and 4 M guanidine hydrochloride. The inset shows spectra recorded for 8-(methylthio)riboflavin in 98% chloroform plus 2% DMSO (curve 1), 98% water plus 2% DMSO (curve 2, dotted line), 70% DMS0 plus 30% water (curve 3), and 100% DMSO (curve 4). (Panel B) Curve 1 is the absorption spectrum of 8-MeSFAD radical bound to photolyase. Curve 2 was recorded after denaturation of the radical form with guanidine hydrochloride. Buffer composition was the same as specified for curves 1 and 2 in panel A, respectively.

8-MeSFAD at 482 nm ($\epsilon_{482} = 36.8 \times 10^3$ M^{-1} cm^{-1}) is larger than the corresponding free flavin in aqueous solution while the converse is true with flavinylated enzyme.

Free 8-(methylthio)riboflavin in chloroform exhibits two peaks ($\lambda_{max} = 480, 454$ nm) (Figure 7A, inset, curve 1), similar to that observed for 8-MeSFAD bound to photolyase. In contrast, the spectral properties of flavinylated enzyme resemble those observed with free 8-(methylthio)riboflavin in 70% DMSO (Figure 7A, inset, curve 3). The results show that 8-(alkylthio)FAD is shielded from solvent when bound covalently or noncovalently to photolyase. However, the flavin environment is clearly affected by covalent attachment.

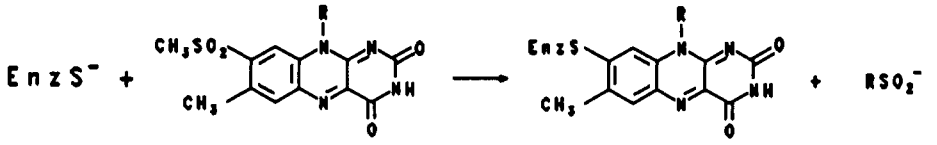
Formation of Photolyase-Bound 8-(Methylthio)FAD Radical. Reaction of 8-MeSFAD enzyme with dithionite converts the oxidized enzyme to the fully reduced state (data not shown). Removal of excess dithionite under aerobic conditions results in the isolation of a blue enzyme form (0.87 mol of flavin/mol of enzyme). The blue color is due to an air-stable, neutral flavin radical as judged by its visible absorption spectrum (Figure 7B, curve 1) and the signal observed in electron spin resonance studies (Rustandi and Jorns, unpublished data). The radical exhibits an intense band at 630 nm with a shoulder at 585 nm plus four additional peaks at shorter wavelengths (484, 402, 384, and 334 nm). The absorption of the radical at 484 nm is quite low as compared with the oxidized enzyme which exhibits an intense peak at 482 nm. An extinction coefficient at 630 nm was calculated ($\epsilon_{630} = 14.4 \times 10^3$ M^{-1} cm^{-1}), assuming 100% conversion to the radical. The stability of the radical is lost upon enzyme denaturation which results in immediate conversion to oxidized 8-MeSFAD (Figure 7B, curve 2). The aerobic stability of photolyase-bound 8-MeSFAD radical is similar to that observed for the FAD radical in native enzyme (Jorns et al., 1984).

Dimer Repair with Photolyase containing 8-(Methylthio)-FAD. Photolyase-bound 8-MeSFAD radical was reduced with dithionite, and the fully reduced enzyme was assayed under anaerobic conditions in DTT-free assay buffer. The rate of dimer repair was linear with respect to the light dose until all the substrate was consumed (no initial lag). The observed rate was 95% of that obtained with dithionite-reduced native enzyme under the same conditions.

A 2-min lag was observed in assays initiated by adding the radical form of 8-MeSFAD enzyme to aerobic reaction mixtures containing DTT. The lag was followed by a linear repair rate similar to that observed in anaerobic assays with fully reduced enzyme. The lag is attributed to an *in situ* photoreduction of the inactive radical form.

A very pronounced lag (no dimer repair detectable during the first 5 min) was seen when DTT-containing aerobic assays were initiated with the oxidized form of 8-MeSFAD enzyme.

Scheme 1



The results suggest that *in situ* photoreduction is more difficult with oxidized than with the radical form of 8-MeSFAD enzyme, similar to that observed with FAD enzyme (Jorns et al., 1990).

Effect of Substrate on the Aerobic Stability of Photolyase-Bound Fully Reduced 8-(Methylthio)FAD. The radical form of 8-MeSFAD enzyme was mixed with UV-irradiated oligo-$(dT)_{18}$ (2.3 mol of dimer/mol of enzyme) and converted to fully reduced enzyme by photoreduction under anaerobic conditions. No dimer repair occurred during photoreduction since the reaction was conducted using red light which excites the radical but not the fully reduced flavin. Reoxidation of a control sample of fully reduced enzyme (no substrate) to the radical level was complete in 10 min. In contrast, no reoxidation of fully reduced enzyme was observed in the presence of substrate after incubation for 18 h at 8 °C. The reduced enzyme–substrate complex was then exposed to photoreactivating light for 4 min. Reoxidation to the radical level was observed within 12 min after dimer repair. The results demonstrate that substrate causes a dramatic increase in the aerobic stability of fully reduced 8-MeSFAD enzyme, similar to that observed with native enzyme (Jordan & Jorns, 1988).

Table 1: Properties of Photolyase Preparations Containing Various Modified Flavins

		activity		aerobic stability[a]		
flavin	attachment	Fl_{ox}	FlH_2	$FlH^•$	FlH_2	FlH_2•S
FAD (native enzyme)	noncovalent	0	100%	stable	+	+++
8-(cysteinyl)FAD	covalent	0	15	nd	labile	–
8-$MeSO_2$FAD	noncovalent	0	6.5	nd	++	–
8-MeSFAD	noncovalent	0	95	stable	+	+++

[a] Aerobic stability of photolyase-bound flavin radical ($FlH^•$) of fully reduced flavin in the absence (FlH_2) or presence of substrate (FlH_2•S) as reported in this paper except for native enzyme (Jordan & Jorns, 1988; Horns et al., 1984): + = oxidation of FlH_2 to $FlH^•$ complete in 10–30 min; ++ = oxidation of FlH_2 to Fl_{ox} complete in 4–6 h; +++ = no reoxidation detected after at least 1 h; nd = $FlH^•$ not detected during flavin reduction and/or reoxidation; – = not determined.

DISCUSSION

In this paper we show that 8-$MeSO_2$FAD reacts with a single cysteine residue (Cys293) in *E. coli* DNA photolyase to yield a flavinylated protein which retains the ability to repair pyrimidine dimers and to bind its second chromophore (5,10-CH^+-H_4folate) (Scheme 1).

Figure 3 shows the domain structure of *E. coli* photolyase as deduced by comparison with results obtained in limited proteolytic digestion studies with the homologous yeast photolyase (Malhotra et al., 1992). The flavin domain comprises the C-terminal half of the protein. This region is highly conserved among members of the two different classes of photolyases which all contain reduced FAD as the reaction center chromophore. Sequence homology in the N-terminal half of the protein, which binds one of the two known antenna chromophores (5,10-CH^+-H_4folate or a 5-deazaflavin derivative), is more limited and tends to be restricted to members of the same class. The flavinylated cysteine, Cys293, lies within the flavin domain, between two highly conserved tryptophans (W278, W307) which appear to be near to the flavin and substrate binding sites (Li & Sancar, 1990; Li et al., 1991).

These results and those obtained in FAD competition experiments indicate that 8-(cysteinyl)FAD in flavinylated photolyase is likely to be at or near the normal flavin binding site. Nevertheless, several observations suggest that the orientation of Cys293 with respect to the C(8) position in FAD is such that a significant perturbation is necessary to accommodate covalent bond formation. The effect of covalent bond formation is most clearly seen by comparing the properties of flavinylated enzyme with enzyme containing noncovalently bound 8-MeSFAD (Table 1). 8-MeSFAD-reconstituted enzyme exhibits nearly the same activity as native enzyme whereas only 15% activity is retained with flavinylated enzyme. The 8-MeSFAD radical is stable under aerobic conditions when bound to photolyase, similar to the FAD radical in native enzyme. The aerobic stability of enzyme-bound fully reduced 8-MeSFAD, although modest, is dramatically increased in the enzyme•substrate complex, again similar to that observed with fully reduced FAD in native enzyme (Jordan & Jorns, 1988). In contrast, radical formation with flavinylated enzyme is not detected during flavin reduction or reoxidation, and the fully reduced flavin is labile under aerobic conditions. Model studies show that the visible absorption spectra of oxidized 8-(alkylthio)flavins are quite sensitive to changes in solvent, suggesting that these derivatives can serve as a useful probe of flavin environment in proteins. The spectral properties of flavinylated and 8-MeSFAD-reconstituted enzyme show that the flavin in both preparations is shielded from solvent but that the environment of the 8-(alkylthio)flavin is affected by covalent attachment. Similarly, although flavinylation does not interfere with pterin binding, the absorption maximum of the bound pterin is hypsochromically shifted as compared with native enzyme.

The results obtained with 8-MeSFAD-reconstituted enzyme show that replacing the 8-methyl group in FAD with an 8-methylthio group does not affect the ability of the flavin to function as a good structural and functional analogue for FAD in photolyase. Very different results are obtained when the 8-methyl group is replaced with a methylsulfonyl moiety. The properties of enzyme containing noncovalently bound 8-$MeSO_2$-FAD (see Table 1) were investigated by reconstituting the apoenzyme with the fully reduced flavin which does not undergo covalent attachment. Dimer repair was observed with enzyme containing noncovalently bound 8-$MeSO_2$FAD, but the rate (6.5%) was even lower than observed with flavinylated enzyme. Enzyme-bound fully reduced 8-$MeSO_2$-FAD is fairly stable against air oxidation as compared with normal FAD or 8-MeSFAD. Unlike the latter preparations, a flavin radical was not detected during reoxidation of 8-$MeSO_2$FAD. Covalent attachment was observed after reoxidation of fully reduced 8-$MeSO_2$FAD enzyme, but the reaction was quite slow and probably involved dissociation/rebinding of oxidized 8-$MeSO_2$FAD.

It is not clear whether the different properties observed with photolyase containing 8-$MeSO_2$FAD reflect a direct effect of the 8-substituent on flavin reactivity or an indirect

effect of the greater bulk of the methylsulfonyl group. 8-$MeSO_2$FMN appears to bind normally to flavodoxin, a protein where the 8-position in FMN is relatively unhindered and accessible to solvent (Raibekas et al., 1993). On the other hand, steric crowding near C(8) in FAD bound to pig lipoamide dehydrogenase may explain why noncovalent binding in the normal orientation is not observed with 8-$MeSO_2$FAD. 8-$MeSO_2$FAD does covalently attach to a cysteine residue near the normal FAD binding site in lipoamide dehydrogenase, but the attachment requires a 180° flip of the isoalloxazine ring as compared with native enzyme (Raibekas & Jorns, 1994).

In summary, we show that Cys293, a residue in the flavin domain of *E. coli* photolyase, becomes covalently linked to the 8-position of FAD during reconstitution with oxidized 8-$MeSO_2$FAD. Although Cys293 in the native structure is probably not in the optimal orientation for nucleophilic attack at C(8), the adjustment needed for covalent bond formation is not sufficient to grossly interfere with the enzyme's ability to repair DNA or to interact with its antenna chromophore.

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