Stereospecificity of Folate Binding to DNA Photolyase from *Escherichia coli*†

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ABSTRACT: DNA photolyase from *Escherichia coli* contains folate ([6*S*]-5,10-CH^+-H_4Pte(Glu)$_{n=3-6}$) and reduced FAD. The folate chromophore acts as an antenna, harvesting light energy which is transferred to the reduced flavin where DNA repair occurs. The folate binding stereospecificity of the enzyme was investigated by reconstituting the apoenzyme with [6*R*,*S*]-5,10-CH^+-H_4folate and reduced FAD. The isomer composition of [*methyl*-^{3}H]-5-CH_3-H_4folate, released into solution upon reduction of the reconstituted enzyme with [^{3}H]$NaBH_4$, was analyzed by enzymatic and chiral chromatographic methods. Both methods showed that the reconstituted enzyme contained nearly equimolar amounts of [6*R*]- and [6*S*]-5,10-CH^+-H_4folate.

Exposure of DNA to ultraviolet light results in the formation of cyclobutane dimers between adjacent pyrimidine residues, a process which has been associated with mutagenesis, carcinogenesis, and cell death. The damage can be repaired by DNA photolyase in a rather unique enzymatic reaction where visible light acts as a second substrate. The catalytically active form of all known photolyases contains fully reduced FAD,[1] probably in the anionic state ($FADH^-$). The enzymes also contain an additional chromophore which is either a folate or an 8-hydroxy-5-deazaflavin derivative, a feature which serves to distinguish two classes of photolyases (Jorns, 1990).

DNA photolyase from *Escherichia coli* contains a folate, [5,10-CH^+-H_4Pte(Glu)$_{n=3-6}$], as its second chromophore. The folate acts as an antenna, harvesting light energy which is then transferred to $FADH^-$. Repair is probably initiated by the transfer of an electron from the reduced flavin singlet ($FADH^-$*) to the dimer (T⟨⟩T), generating a neutral flavin radical ($FADH^{\cdot}$) and an unstable dimer anion radical (T⟨⟩$T^{\cdot-}$) which can rapidly monomerize. Subsequent back-electron-transfer regenerates $FADH^-$. $FADH^-$* can be formed by excitation of $FADH^-$ or via energy transfer from the folate singlet (Jordan & Jorns, 1988; Jorns et al., 1987b, 1990; Lipman & Jorns, 1992; Ramsey et al., 1992; Okamura et al., 1991; Kim et al., 1991; Rustandi & Jorns, 1995).

Direct evidence for singlet−singlet energy transfer from folate to $FADH^-$ was obtained from fluorescence quantum yield experiments (Lipman & Jorns, 1992). These studies showed that interchromophore energy transfer is highly efficient in native enzyme (E_{ET} = 0.92) but decreases 2-fold in enzyme reconstituted with [6*R*,*S*]-5,10-CH^+-H_4folate. The decreased efficiency of interchromophore energy transfer in reconstituted enzyme might be due to the substitution of the polyglutamate moiety in the natural folate with a monoglutamate or because the synthetic folate was racemic. In this paper the folate binding stereospecificity of photolyase was investigated by analysis of the isomers of 5,10-CH^+-H_4folate that are bound to reconstituted enzyme.

EXPERIMENTAL PROCEDURES

Materials. [6*R*,*S*]-5-CHO-H_4folate (Ca^{2+} salt), FAD, L-homocysteine thiolactone, *S*-adenosyl-L-methionine, DTT, hydroxycobalamin, and human serum albumin were purchased from Sigma. L-Homocysteine thiolactone was converted to L-homocysteine prior to use by alkaline hydrolysis (Weissbach et al., 1963). [6*R*,*S*]-H_4folate·3HCl was purchased from Dr. B. Schircks Laboratories.

Preparation of 5,10-Methenyltetrahydrofolate. [6*R*,*S*]-5-CHO-H_4folate was converted to 5,10-CH^+-H_4folate according to the method described by Rabinowitz (1963). Briefly, [6*R*,*S*]-5-CHO-H_4folate (50 mg) was suspended in 4 mL of 1.0 M β-mercaptoethanol and dissolved by adjusting the pH to 1.5 with 6 N HCl (about 3 drops). The sample was incubated for 1 h at room temperature and then for 48 h at 4 °C. The 5,10-CH^+-H_4folate precipitate was collected by centrifugation and washed with 2 mL of HPLC grade acetonitrile. The pellet was dissolved in 4 mL of HPLC grade methanol, divided into 1-mL aliquots, evaporated to dryness using a Savant Speed-Vac, and then stored at −20 °C. Stock solutions were made by dissolving the sample in 0.01 N HCl.

Holoenzyme and Reconstituted Enzyme Preparation. E. coli DNA photolyase was purified as previously described (Jorns et al., 1987a). Apophotolyase was prepared and reconstituted with $FADH_2$ and [6*R*,*S*]-5,10-CH^+-H_4folate as described by Jorns et al. (1990). The reconstituted enzyme (1.4×10^{-4} M) was stored at −80 °C in storage buffer [50 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 1.0 mM

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[1] Abbreviations: FAD, flavin adenine dinucleotide; $FADH^{\cdot}$, blue neutral FAD radical; $FADH^-$, fully reduced FAD anion; T⟨⟩T, thymine dimer; 5,10-CH^+-H_4Pte(Glu)$_n$, 5,10-methenyltetrahydropteroylpolyglutamate; 5,10-CH^+-H_4folate, 5,10-methenyltetrahydrofolate; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; 5-CHO-H_4folate, 5-formyltetrahydrofolate; 10-CHO-H_4folate, 10-formyltetrahydrofolate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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EDTA, 10 mM dithiothreitol (DTT), and 50% glycerol]. Enzyme concentration was calculated on the basis of FADH$^{\cdot}$ absorption at 580 nm ($\epsilon_{580} = 4.8 \times 10^{-3}$ M^{-1} cm^{-1}) (Wang & Jorns, 1989). The concentration of enzyme-bound 5,10-CH^{+}-H$_4$folate was estimated on the basis of its absorption at 380 nm after correcting for the contribution due to FADH$^{\cdot}$, as previously described (Jorns et al., 1990). The molar ratio of folate to flavin was 1.03. Absorption spectra were recorded at 5 °C using a Perkin-Elmer Lambda 3B spectrometer.

Reduction of Free or Photolyase-Bound 5,10-CH^{+}-H$_4$folate with [^{3}H]NaBH$_4$. 5,10-CH^{+}-H$_4$folate in reconstituted enzyme (4.2 μM) was converted to [*methyl*-^{3}H]-5-CH$_3$-H$_4$folate by reduction with 16.8 mM [^{3}H]NaBH$_4$ (120 mCi/mmol) in storage buffer containing 15% glycerol. [*methyl*-^{3}H]-5-CH$_3$-H$_4$folate is released from the enzyme and recovered in the filtrate obtained after microfiltration (Centricon −10). Borohydride reduction of free 5,10-CH^{+}-H$_4$folate (4.2 μM) to [*methyl*-^{3}H]-5-CH$_3$-H$_4$folate (~95% conversion) was conducted in citrate buffer (50 mM sodium citrate, pH 6.0, containing 50 mM NaCl, 10 mM DTT, and 1.0 mM EDTA) under otherwise identical conditions.

Enzyme-derived and control samples of [*methyl*-^{3}H]-5-CH$_3$-H$_4$folate were treated identically in all subsequent steps. The samples were applied to a Sep-Pak Plus tC$_{18}$ cartridge, eluted with methanol, evaporated to dryness, redissolved in citrate buffer, and injected onto a Rainin Microsorb C$_{18}$ 80-225-C5 column. The column was developed using the following elution profile (flow rate = 0.5 mL/min): 10 min of isocratic elution with 10% methanol adjusted to pH 2.2 with phosphoric acid, a 10-min linear gradient to 30% methanol (pH 2.2), and 20 min of isocratic elution with 30% methanol (pH 2.2). [*methyl*-^{3}H]-5-CH$_3$-H$_4$folate, detected by its absorbance at 290 nm or its tritium content, eluted at about 25 min. Peak fractions were pooled and evaporated. Some decomposition was noted after storage at −80 °C, possibly due to residual phosphoric acid. Therefore, both enzyme-derived and control samples were subjected to a second HPLC purification using a Vydac 218TP5405 (C$_{18}$, 5 μm, 4.6 × 50 mm) reversed-phase column and the following elution profile (flow rate = 1.0 mL/min): 10 min of isocratic elution with water containing 0.1% trifluoroacetic acid, a 5-min gradient to 10% methanol (plus 0.1% trifluoroacetic acid), and 5 min of isocratic elution with 10% methanol (plus 0.1% trifluoroacetic acid). Fractions containing [*methyl*-^{3}H]-5-CH$_3$-H$_4$folate were pooled and evaporated to dryness. These samples were stable when stored at −80 °C. The same specific activity was observed for enzyme-derived and control samples (62 700 cpm/nmol). For analysis, the samples were redissolved in 8 mM sodium ascorbate.

Stereochemical Analysis of [methyl-^{3}H]-5-CH$_3$-H$_4$folate with Methionine Synthase. Recombinant methionine synthase from *E. coli* was isolated as previously described (Banerjee et al., 1989). The methionine synthase assay was conducted as previously described (Drummond et al., 1993) except that reaction conditions were adjusted to ensure complete reaction with all available [6*S*]-5-CH$_3$-H$_4$folate. Briefly, 0.3−0.4 nmol of [*methyl*-^{3}H]-5-CH$_3$-H$_4$folate (enzyme-derived or control sample) was added to 985 μL of 100 mM potassium phosphate buffer, pH 7.2, containing 25 mM DTT, 19 μM *S*-adenosyl-L-methionine, 100 μM [6*R*,*S*]-5-CH$_3$-H$_4$folate (unlabeled), 50 μM hydroxycobalamin, and 10 μg of methionine synthase (~5 milliunits). After a 5-min incubation at 37 °C, the reaction was initiated by adding homocysteine (5 μL) to a final concentration of 500 μM. After a 30-min incubation at 37 °C, the reaction mixture was filtered through a small Bio-Rad AG1-X8 column to remove unreacted [6*R*]-5-CH$_3$-H$_4$folate. Scintillation fluid was added to the eluate, and the samples were counted. For each sample, duplicate incubations were conducted except that methionine synthase or the column step was omitted. The latter reactions were used to determine total radioactivity. The counts observed in the AG1-X8 eluates from reaction mixtures lacking methionine synthase were used to correct for background and any radioactivity in the original sample that did not adsorb to the AG1-X8 column.

Stereochemical Analysis of [methyl-^{3}H]-5-CH$_3$-H$_4$folate Using Chiral Chromatography. The chromatography system consisted of a Nucleosil 7μ diol column that had been derivatized with human serum albumin according to the procedure of Domenici et al. (1990). The column was eluted at 0.7 mL/min at 32 °C with 50 mM potassium phosphate buffer, pH 6.8/1-propanol (93:7) and was put into a deoxygenated state by an initial injection of 25 μL of 1 M sodium dithionite just prior to sample analysis. A Waters 996 photodiode array UV spectrometer was used for detection. A standard sample of racemic [6*R*,*S*]-5-CH$_3$-H$_4$folate was freshly generated by the procedure of Gupta and Huennekens (1967), starting from a preparation of [6*R*,*S*]-H$_4$folate·3HCl. The crude reaction mixture of [6*R*,*S*]-5-CH$_3$-H$_4$folate was used directly after acidification. The elution order of the isomers was established by using [6*S*]-5-CH$_3$-H$_4$folate. The [6*S*]-isomer was similarly prepared using [6*S*]-H$_4$folate (Bailey et al., 1992) instead of a racemic mixture.

RESULTS

Reduction of Free or Photolyase-Bound 5,10-CH^{+}-H$_4$folate with [^{3}H]NaBH$_4$. Apophotolyase was reconstituted with [6*R*,*S*]-5,10-CH^{+}-H$_4$folate plus FADH$_2$. The fully reduced flavin was oxidized to a neutral radical during isolation of the reconstituted enzyme which contained equimolar amounts of FADH$^{\cdot}$ and 5,10-CH^{+}-H$_4$folate. For stereochemical analysis, the reconstituted enzyme was reduced with [^{3}H]NaBH$_4$. In this reaction, 5,10-CH^{+}-H$_4$folate is converted to [*methyl*-^{3}H]-5-CH$_3$-H$_4$folate, which is released from the enzyme and easily separated from the protein by microfiltration (Jorns et al., 1987b; Hamm-Alvarez et al., 1989). In a control reaction, free [6*R*,*S*]-5,10-CH^{+}-H$_4$folate was reduced with [^{3}H]NaBH$_4$ under similar conditions except that a somewhat lower pH (6.0 *versus* 7.4) was used to minimize decomposition of the protein-free chromophore. Enzyme-derived and control samples of [*methyl*-^{3}H]-5-CH$_3$-H$_4$folate were purified by the same procedure, as detailed in Experimental Procedures.

Stereochemical Analysis of [methyl-^{3}H]-5-CH$_3$-H$_4$folate with Methionine Synthase. Methionine synthase is stereospecific for [6*S*]-5-CH$_3$-H$_4$folate (Larrabee et al., 1963; Fontecilla-Camps et al., 1979). The enzyme catalyzes the transfer of the 5-methyl group from [6*S*]-5-CH$_3$-H$_4$folate to homocysteine, forming methionine (eq 1).

$$[6S]\text{-}[\textit{methyl}\text{-}^3\text{H}]\text{-}5\text{-}CH_3\text{-}H_4\text{folate} + \text{homocysteine} \rightarrow [6S]\text{-}H_4\text{folate} + [\textit{methyl}\text{-}^3\text{H}]\text{methionine} \quad (1)$$

The amount of tritium label incorporated into [*methyl*-^{3}H]-

Table 1: Stereochemical Analysis of Various 5-CH_3-H_4folate Samples

source	[6S]-isomer (%) methionine synthase analysis[a]	chiral chromatography[b]
[^{3}H]$NaBH_4$ reduction of photolyase-bound 5,10-CH^+-H_4folate	51.2 ± 0.65	55
[^{3}H]$NaBH_4$ reduction of free 5,10-CH^+-H_4folate	70.7 ± 1.18	74

[a] Results are the average of four determinations (±SE). [b] A single analysis was performed on each sample. The integration of the rather broad peaks may be associated with a 5% possible error.

methionine, determined after reaction mixtures were filtered to remove any unreacted [6*R*]-[*methyl*-^{3}H]-5-CH_3-H_4folate, was used to estimate the [6*S*]-isomer content in enzyme-derived and control samples of [*methyl*-^{3}H]-5-CH_3-H_4folate. The results show that the photolyase sample had a 1:1 isomer ratio. However, the control sample appeared to contain about 70% [6*S*]-isomer (Table 1).

Stereochemical Analysis of [methyl-^{3}H]-5-CH_3-H_4folate Using Chiral Chromatography. Chromatographic analyses were conducted using an HPLC column prepared by covalently attaching human serum albumin to a diol column, a procedure previously shown to generate a matrix capable of resolving isomeric mixtures of biological interest, including folates (Domenici et al., 1990). For these studies, a standard sample of [6*R*,*S*]-5-CH_3-H_4folate was freshly synthesized by the procedure of Gupta and Huennekens (1967), starting from a commercial [6*R*,*S*]-H_4folate preparation that was previously established to be racemic (Bailey et al., 1992; S. W. Bailey, unpublished results). The crude reaction mixture of [6*R*,*S*]-5-CH_3-H_4folate was used without purification, which might alter the *R*/*S* ratio. The major impurities eluted early as two minor peaks (R_E = 5.5 and 9.5 min) that did not interfere with the analysis of the peaks due to the [6*S*]- and [6*R*]-isomers of 5-CH_3-H_4folate, which eluted later (R_E = 14.2 and 18.2 min) (Figure 1A). The elution profiles obtained for the [6*S*]- and [6*R*]-isomers in photolyase-derived and control samples of [*methyl*-^{3}H]-5-CH_3-H_4folate are shown in panels B and C, respectively, in Figure 1. With all samples, the absorption spectra of the peaks of both isomers were identical and consistent with that expected for 5-CH_3-H_4folate at pH 6.8 (λ_{max} = 289 ± 1 nm). The spectral shape remained constant over all of each peak, indicating that any co-eluting impurities were minimal. The only exception is a small bump eluting just prior to the [6*R*]-isomer in the crude reaction mixture. In all samples, an unexpected forward shoulder is observed on the [6*S*]-isomer peak and is probably explained by a small void that was found on opening the column after the runs were completed.

Using integrations similar to that which gave a 50:50 ratio with the standard sample of [6*R*,*S*]-5-CH_3-H_4folate, the photolyase-derived sample showed 55% [6*S*]-5-CH_3-H_4folate, while the control sample gave 74% [6*S*]-isomer. The results are consistent with those obtained in analyses with methionine synthase (Table 1).

DISCUSSION

The apoenzyme of *E. coli* photolyase was reconstituted with [6*R*,*S*]-5,10-CH^+-H_4folate and $FADH_2$. The folate

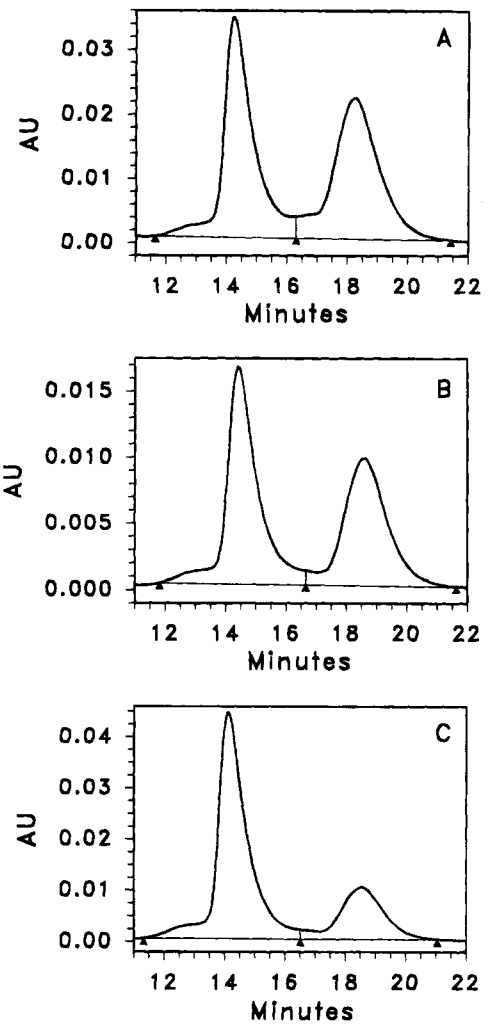

FIGURE 1: Separation of [6*S*]- and [6*R*]-isomers of 5-CH_3-H_4folate on a human serum albumin-derivatized HPLC column. Panel A shows the isomer elution profile obtained at 290 nm for a standard sample of [6*R*,*S*]-5-CH_3-H_4folate. The early portion of the chromatogram (not shown) contains minor peaks [at 5.5 and 9.5 min (unreacted H_4folate)] due to impurities in the crude reaction mixture. Panels B and C show the isomer elution profiles obtained for photolyase-derived and control samples of [*methyl*-^{3}H]-5-CH_3-H_4-folate. These samples were prepared in 8 mM sodium ascorbate which eluted as a large peak at the solvent front (not shown). The integration regions are indicated.

binding stereospecificity of the enzyme was investigated by analysis of the isomers of [*methyl*-^{3}H]-5-CH_3-H_4folate that are released into solution upon reduction of the reconstituted enzyme with [^{3}H]$NaBH_4$. Enzymatic analyses were conducted using methionine synthase, which is specific for [6*S*]-5-CH_3-H_4folate (Larrabee et al., 1963; Fontecilla-Camps et al., 1979). HPLC analyses were conducted using a human serum albumin-derivatized column where the natural [6*S*]-isomer of 5-CH_3-H_4folate elutes first, consistent with a report by Mader et al. (1994) on the higher affinity of free human serum albumin for the unnatural [6*R*]-isomer. The observed elution order parallels that previously observed using this matrix and 5-CHO-H_4folate (Domenici et al., 1990) but is opposite that found with 5-CH_3-H_4folate on a bovine serum albumin-based column where the [6*R*]-isomer elutes first (under somewhat different elution conditions) (Wainer & Stiffin, 1988). An analogous reversal of elution order, observed with warfarin isomers, has been attributed to differences in the binding specificity of human *versus* bovine

serum albumin (Domenici et al., 1990; Wainer & Stiffin, 1988).

Both the enzymic and the chromatographic methods showed that the photolyase-derived sample of [*methyl*-^{3}H]-5-CH_3-H_4folate was completely racemic. Unexpectedly, a control sample of [*methyl*-^{3}H]-5-CH_3-H_4folate, prepared by reacting free [6*R*,*S*]-5,10-CH^+-H_4folate with [^{3}H]$NaBH_4$, was partially enriched (~70%) with respect to the [6*S*]-isomer. It is possible that the commercial [6*R*,*S*]-5-CHO-H_4folate preparation used to synthesize 5,10-CH^+-H_4folate was not truly racemic. Another possibility is that enrichment with the [6*S*]-isomer occurred during precipitation of 5,10-CH^+-H_4folate from HCl, a step used in the isolation of the compound. Isomer enrichment can occur during purification of tetrahydrofolates, as strikingly illustrated by the isolation of the [6*S*]-isomer of 5-CHO-H_4folate by fractional crystallization of a racemic mixture of its calcium salt from water (Cosulich et al., 1952).

The results clearly show that *E. coli* photolyase can bind either the [6*R*]- or the [6*S*]-isomer of 5,10-CH^+-H_4folate. The enzyme may preferentially bind the nonphysiological [6*R*]-isomer, as judged by the 1.7-fold larger amount found in enzyme-derived *versus* control samples of [*methyl*-^{3}H]-5-CH_3-H_4folate, both prepared starting with the same lot of 5,10-CH^+-H_4folate. The unnatural [6*S*]-isomer of 10-CHO-H_4folate is tightly bound to chicken liver glycinamide ribonucleotide transformylase where it acts as a competitive inhibitor with a K_i value nearly an order of magnitude lower than the K_m value observed for the [6*R*]-isomer (Smith et al., 1981). Similarly, the unnatural [6*S*]-isomer of 5,10-CH_2-H_4folate is a competitive inhibitor of thymidylate synthetase from *Lactobacillus casei* ($K_i \sim 4K_{m\ (\text{natural isomer})}$) (Leary et al., 1974), although the enzyme from other sources will bind only the natural isomer (Bisson & Thorner, 1981; Meek et al., 1985). In the case of photolyase, it is not yet known whether the unnatural isomer can function in catalysis.

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