The Mechanism and Substrate Specificity of the NADPH:Flavin Oxidoreductase from Escherichia coli*

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The NAD(P)H:flavin oxidoreductase from Escherichia coli, Fre, is a monomer of 26.2 kDa that catalyzes the reduction of free flavins by NADPH or NADH. Overexpression in E. coli now allows the preparation of large amounts of pure protein. Structural requirements for recognition of flavins as substrates and not as cofactors were studied by steady-state kinetics with a variety of flavin analogs. The entire isooxazoline ring was found to be the essential part of the flavin molecule for interaction with the polypeptide chain. Methyl groups at C-7 and C-8 of the isooxazoline ring and the N-3 of riboflavin also play an important role in that interaction, whereas the ribityl chain of the riboflavin is not required for binding to the protein. On the other hand, the presence of the 2-OH of the ribityl chain stimulates the NADPH-dependent reaction significantly. Moreover, a study of competitive inhibitors for both substrates demonstrated that Fre follows a sequential ordered mechanism in which NADPH binds first followed by riboflavin. Lumichrome, a very good inhibitor of Fre, may be used to inhibit flavin reductase in E. coli growing cells. As a consequence, it can enhance the antiproliferative effect of hydroxyurea, a cell-specific ribonucleotide reductase inactivator.

Flavins are well known as key prosthetic groups of a large number of redox enzymes named flavoproteins. More recently, protein-free flavins, riboflavin, FMN, or FAD, were also suggested to play, as electron transfer mediators, important biological functions, for example during ferric iron reduction (1-3), activation of ribonucleotide reductase (4, 5), bioluminescence (6, 7), and oxygen activation (8) (Scheme 1).

The reduction of free flavins by reduced pyridine nucleotides NADPH or NADH is not an efficient reaction. The kinetics is slow unless very high nonphysiological concentrations of both reactants are present in the reaction mixture (8). As a consequence, living organisms have evolved enzymes that catalyze the reduction of riboflavin, FMN, and FAD by NADPH and NADH and are called NAD(P)H:flavin oxidoreductases or flavin reductases. It is now well established that such enzymes are present in all microorganisms, including the luminous marine bacteria, and also in mammals (1). A recent study has shown that flavin reductase activities are present in erythrocytes and in various human tissues (liver, heart, kidney, and lung) (9).

In most cases, a single living organism contains multiple flavin reductases different in enzymatic nature and molecular mass. The luminous bacteria, Vibrio harveyi, contains at least three types of FMN reductases (10–14). In Escherichia coli at least two flavin reductases have been isolated. One, named Fre, is a 26.2-kDa enzyme using both NADH and NADPH as electron donors (4), whereas the other is the sulfite reductase, a 780-kDa enzyme using NADPH exclusively (15).

Still very little is known about the structure and the mechanisms of flavin reductases. No three-dimensional structure of such an enzyme is available yet, and only recently were the corresponding genes cloned, sequenced and overexpressed (9–12, 16–19). Only in the cases of E. coli and V. harveyi were the enzymes obtained in a pure form and characterized (4, 13, 15). In spite of such a limited information, it is nevertheless possible to propose a classification of flavin reductases in two groups.

In the first group, enzymes are flavoproteins, using a flavin prosthetic group for electron transfer from NAD(P)H to the flavin substrate. The prototype of this group is the NADPH-specific flavin reductase from V. harveyi (flavin reductase P) (13). It is a monomer of 26 kDa with a tightly bound FMN cofactor and has been recently crystallized. Preliminary x-ray diffraction analysis of the protein has been reported (20). The sulfite reductase of E. coli carries both FAD and FMN as cofactors and thus belongs to this group. This class of enzymes is characterized by ping-pong bisubstrate biproduct reaction mechanisms (14, 21).

In the second group, enzymes do not contain any prosthetic group. The visible spectrum of the protein gives no evidence for a chromophore and excludes the presence of flavins. When added, FMN or FAD does not bind tightly, and the enzyme thus should not be classified as a flavoprotein. The prototype of this group is Fre, the NAD(P)H:flavin oxidoreductase from E. coli (4). It consists of a single polypeptide chain of 233 amino acids, with a molecular mass of 26,212 Da. The NADH-specific FMN reductase enzyme from V. harveyi (flavin reductase D) belongs to this group and actually has significant amino acid sequence homology with Fre (48% identity) as well as a similar molecular weight (10). A number of ferric reductases, for example from Rhodopseudomonas sphaeroıdes, Pseudomonas aeruginosa, or Neisseria gonorrhoeae, which absolutely require free flavins for activity, probably also belong to this group (22, 23).

Such flavin reductases are interesting systems to study in that they utilize flavin only as a substrate and not as a coenzyme. It is thus important to understand how the polypeptide chain accommodates both reduced pyridine nucleotide and flavin to allow the electron transfer to proceed efficiently. It is also important to appreciate the structural basis for the difference in flavin recognition between flavoproteins and flavin reductases in order to delineate the different possible ways of interaction between the flavin and the polypeptide chain in relation with the function.

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In this paper we have used a variety of flavin analogs (substrates and inhibitors) to probe the structural requirements of the flavin binding site of Fre, the NAD(P)H:flavin oxidoreductase from E. coli. Detailed kinetic studies of flavin reduction, in the presence of NADPH, show that the reductase follows a sequential mechanism, in agreement with the absence of a protein-bound mediator. Orders of substrate binding and product release have been determined. It is clearly established that the binding of the flavin occurs mainly through the isoalloxazine ring. In addition, lumichrome was found to be a very good inhibitor of the flavin reductase and to potentiate hydroxyurea as an inhibitor of ribonucleotide reductase and E. coli cell growth.

**Materials and Methods**

Biochemical and Chemical Reagents—Restriction enzymes and DNA modifying enzymes were obtained from Eurogentec. Yeast extract and tryptone were from Biokar Diagnostics. Riboflavin (1), lumiflavin (2), alocixalone (9), lumichrome (10), AMP, NADH, NADPH, NADP+, FMN, and FAD were obtained from Sigma. 3-Bromo-1,2-propanediol, 5-chlorouracil, and riboflavin 5-oxide were obtained from Sigma. 3-Bromo-1,2-propanediol, 5-chlorouracil, and riboflavin 5-oxide were obtained from Sigma. For 3,4-dimethylaniline derivatives have been prepared with different side chains referred to as a for 2-hydroxyethyl (starting compound, 2-bromoethanol), b for 3-hydroxypropyl (starting compound, 3-bromopropanoic acid), and c for 2,3-dihydroxypropyl (starting compound, 3-bromo-1,2-propanediol). For 3,4-dimethylaniline derivatives a, b, and c, a mixture of 3,4-dimethylaniline (9.1 g, 75 mmol), triethylamine (15 cm3), and bromoalcohol (25 mmol) was stirred at 110 °C for 5 h. After cooling and addition of dichloromethane (2 × 100 cm3), the solution was washed with an aqueous Na2CO3 solution (10%; 40 cm3). The aqueous layer was extracted with dichloromethane (200 cm3). The combined organic extracts were dried over MgSO4 and evaporated under reduced pressure. Compound 11a was purified by chromatography on silica gel eluting with a dichloromethane/methanol mixture (98:2) (yield, 70% oil). 1H NMR (80 MHz, CDCl3) δ 7.02 (1H, d, J = 8 Hz, ArH); 6.53 (1H, s, ArH); 6.42 (1H, d, J = 8 Hz, ArH); 3.75 (2H, t, CH2OH or CH3NH); 3.35 (2H, br s, NH and OH); 3.27 (2H, t, CH2NH or CH2OH); 2.25 (8H, s, 2 CH2). 13C NMR (20 MHz, CDCl3) δ 146.5; 137.0; 130.1 (ArCH); 125.5; 115.3 (ArCH); 110.8 (ArCH); 60.6 (CH2OH or CH2NH); 46.3 (CH2NH or CH2OH); 19.7 (CH2); 18.4 (CH3). MS (EI) m/e 165 (88, M+); 164 (100, (M-1)+); 105 (M-NCH2CH2OH)+. Compound 11b was obtained in a pure form after chromatography on silica gel eluting with a dichloromethane/methanol mixture (98:2) or after distillation under reduced pressure (yield, 80% oil). b.p. 107–110 °C (p = 0.1 mm Hg). 1H NMR (300 MHz, CDCl3) δ 6.98 (1H, d, J = 8 Hz, ArH); 6.70 (1H, s, ArH); 6.67 (1H, d, J = 8 Hz, ArH); 3.82 (2H, m, CH2OH or CH2NH); 3.60 (1H, m, NH or OH); 3.40 (1H, br s, OH or NH); 3.22 (2H, m, CH2NH or CH2OH); 2.19 (3H, s, CH3); 2.16 (3H, s, CH3); 1.90 (2H, m, CH2). 13C NMR (75 MHz, CDCl3) δ 146.3; 136.9; 130.0 (ArCH); 125.4; 115.0 (ArCH); 110.6 (ArCH); 61.0 (CH2OH or CH2NH); 42.1 (CH2NH or CH2OH); 31.8 (CH3); 18.8 (CH3). Compound 11c was purified by washing of the crude residue with dichloromethane (yield, 64%), m.p. 100–102 °C. 1H NMR (200 MHz, Me6SO-d6) δ 6.79 (1H, d, J = 8 Hz, ArH); 6.38 (1H, d, J = 2 Hz, ArH); 6.30 (1H, dd, J = 8 Hz, 2 Hz, ArH); 4.90 (1H, t, OH or NH); 4.88 (1H, dd, J = 4.7 Hz, CH2OH); 4.52 (1H, td, J = 5.6 Hz, NH or OH); 3.60 (1H, br s, CH2OH); 3.33 (2H, m, CH2OH or CH2NH); 2.90 (2H, m, CH2NH or CH2OH); 2.08 (3H, s, CH3); 2.04 (3H, s, CH3). 13C NMR (50 MHz, Me6SO-d6) δ 147.1; 136.1; 130.0 (ArCH); 123.0; 114.0 (ArCH); 109.7 (ArCH); 70.1 (CH2OH); 64.1 (CH2OH or CH2NH); 46.8 (CH2NH or CH2OH); 19.8 (CH3); 18.4 (CH3). MS (FAB) m/e 196 (100, (M+1)+); 121 (14, M+1-CH2OHCH2OH+).

For 6-(N-substituted anilinouracil) derivatives 12a, b, and c, compound 11 (21 mmol) was dissolved in water (11c, 30 cm3) or in a 1:1 water/dioxiane mixture (11b, 35 cm3; 11b, 40 cm3). The solution was heated at reflux under argon and stirred during addition of 6-chlorouracil (3 × 100 cm3) to remove unreacted starting compound 11. Aqueous HCl was added to the aqueous layer to reach pH 3. The resulting precipitate was collected by filtration, washed with water, and then crystallized from water. A second fraction of 12a or 12b was obtained after evaporation of the filtrates. The residue was stirred with methanol and filtered. Methanol was evaporated, and the residual solid was crystallized from water (yields: 12a, 50%; 12b, 87%). alocxalone (9) NMR (200 MHz, Me6SO-d6) δ 10.35 (1H, br s, NH); 10.0 (1H, br s, NH); 7.20 (1H, d, J = 8 Hz, ArH); 7.07 (1H, d, ArH); 7.00 (1H, dd, J = 8 Hz, ArH); 5.40 (1H, br s, OH); 4.03 (1H, s, 5-CH); 3.70 (2H, m, 3'-CH or 1'-CH); 3.50 (2H, m, 1'-CH or 3'-CH); 2.22 (6H, s, 2 CH3). 13C NMR (50 MHz, Me6SO-d6) δ 163.6 (C-O); 155.0 (C-O); 151.0 (C6); 140.3; 137.9; 135.7; 130.6 (ArCH); 128.5 (ArCH); 127.4 (ArCH); 85.7 (2 CH2 or 1'-CH or 3'-CH); 53.8 (1'-CH or 3'-CH); 19.3 (CH3). MS (EI) m/e 275 (63, M+); 274 (83, (M-1)+); 105 (M-NCH2CH2OH+). Compound 11b was prepared with different side chains referred to as a for 2-hydroxyethyl (starting compound, 2-bromoethanol), b for 3-hydroxypropyl (starting compound, 3-bromopropanoic acid), and c for 2,3-dihydroxypropyl (starting compound, 3-bromo-1,2-propanediol). The intermediates 11a, 12a, and 13 have been prepared with different side chains referred to as a for 2-hydroxyethyl (starting compound, 2-bromoethanol), b for 3-hydroxypropyl (starting compound, 3-bromopropanoic acid), and c for 2,3-dihydroxypropyl (starting compound, 3-bromo-1,2-propanediol).
NADPH: Flavin Oxidoreductase from E. coli

\[\text{C}_4\text{H}_5\text{N}_2\text{O}_2\text{N}_7 \cdot \text{H}_2\text{O} (334.33) \]
Calculated: C 53.89 H 5.43 N 16.76
Found: C 53.79 H 5.24 N 16.48

Compound 6 (N-Phosphonate)—A suspension of compound 4 (50 mg; 0.16 mmol) was heated at 110°C in pyridine (10 cm³) under argon in the dark until dissolution. The solution was then allowed to cool at 30°C. After the addition of phosphoric acid (54 mg; 0.66 mmol) and 2,4,6-trisopropylbenzenesulfonyl chloride (48 mg; 0.16 mmol), the mixture was stirred for 15 h. Compound 6 was collected by filtration and washed with diethyl ether. It was chromatographed on a C18 reversed phase elution with water/methanol mixtures, m.p. 272°C (dec.) [lit. (25) 275°C (dec.)].

**N**-[Methylisobioflavin 7—Riboflavin (0.68 g; 1.81 mmol) was heated under argon atmosphere at 100°C in N,N-dimethylformamide (250 cm³) with stirring until dissolution. The resulting solution was allowed to cool at 40°C before addition of K₂CO₃ (5 g; 36 mmol) and then at room temperature, before the addition of methyl iodide (20 cm³). The mixture was stirred for 4.5 h. The reaction mixture was filtered. The filtrate was evaporated to dryness under reduced pressure, and the residue was washed with diethyl ether and then dissolved in methanol. The insoluble material was removed by filtration, and the methanol was evaporated under reduced pressure. The residue was washed with water and then crystallized from methanol to afford 7 (yield, 66%). For biological experiments, a fraction of 7 was chromatographed on a C18 reversed phase elution with water/methanol mixtures, m.p. 272°C (dec.) [lit. (25) 275°C (dec.)].

**10-Methylisolaaxazine 8**—For 6-(N-methylinloinoruluracil 14, 6-chlorouracil (4.7 g; 32 mmol) was added with stirring to a solution of N-methylinolinuracil (10.3 g; 96 mmol) in an ethanol/water mixture (2:1; 150 cm³) heated to reflux under argon. The mixture was refluxed for 24 h and then cooled. A first fraction of 14, which crystallized from the solution, was obtained by filtration. The filtrate was evaporated under reduced pressure, and the unreacted N-methylinolinuracil was removed by coevaporation with water. The residue was washed with diethyl ether and then crystallized from an ethanol/water mixture (75:25) (yield, 63%). m.p. 287–289°C. [lit.] H NMR (300 MHz, CDC₁₇) δ 7.60 (2H, br, s, NH); 7.50 (2H, br, s, CH); 7.38 (2H, br, s, N–CH₃); 7.20 (2H, s, CH); 4.93 (2H, br, s, CH); 3.50 (2H, br, s, CH); 2.60 (2H, br, s, CH). For 10-methylisolaaxazine 15, sodium nitrate was added (0.7 g; 10.1 mmol) to a solution of 14 (0.47 g; 2.34 mmol) in acetic acid (20 cm³) in the dark. The resulting solution was stirred for 2 h at room temperature, and then water (40 cm³) was added. The mixture was kept at 4°C overnight. Compound 15 was collected by filtration and washed with water and then dried (yield, 87%). m.p. > 300°C, lit. (24) > 360°C. For 10-methylisolaaxazine 8, a mixture of compound 15 (0.15 g; 0.64 mmol) and N,N-dimethylformamide (50 cm³) was refluxed for 1 h under argon. The solvent was removed under reduced pressure, and the residue was washed with diethyl ether and then ethanol. It was crystallized from acetonitrile. A second crystallized material was obtained from acetonitrile compound 8 (yield, 66%). [lit.] H NMR (200 MHz, MeSO₄-d₆) δ 11.40 (1H, br, s, NH); 8.12 (1H, d, ArH); 7.94 (2H, m, 2 ArH); 7.64 (1H, m, ArH); 3.97 (3H, s, CH₃). MS (DCI, NH₄⁺/isobutane) m/z 229 (100, M–1)⁺.

**Riboflavin 5-Oxide 13d**—This compound was prepared according to the method described by Yoneda et al. (24). m.p. 225°C (dec.) [lit. (24) 225°C (dec.)].

\[\text{C}_4\text{H}_5\text{N}_2\text{O}_2 \cdot 1/4\text{CH}_2\text{CO}_2\text{H} (243.22) \]
Calculated: C 56.97 H 3.73 N 23.04
Found: C 56.60 H 3.66 N 23.10

The abbreviations used are: NBR, 3-nitrobenzyl alcohol; IPTG, isopropyl β-D-thiogalactopyranoside.

\[\text{C}_4\text{H}_5\text{N}_2\text{O}_2\] (300.33)
Calculated: C 59.99 H 5.37 N 18.76
Found: C 59.57 C 5.58 H 18.17

\[\text{C}_4\text{H}_5\text{N}_2\text{O}_2\] (300.33)
Calculated: C 59.99 H 5.37 N 18.76
Found: C 59.57 C 5.58 H 18.17

5: m.p. 300°C (dec.). [lit.] H NMR (200 MHz, MeSO₄-d₆) δ 11.29 (1H, NH); 8.02 (1H, s, ArH); 7.47 (1H, m, 2O–CH₂); 4.72 (2H, 2, 3–CH₂ or C–CH₃); 3.55 (2H, br, s, 3–CH₂ or C–CH₃); 2.41 (3H, s, CH₃); 2.30 (1H, br, s, 2–CH₂). [lit.] C14H18N2O2 (301.34, 311.04 M–1) + : 243 (15), 187 (17), MS (FAB [+] glycerol) m/z 302 (16, M–1)⁺.

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**References**

1. The abbreviations used are: NBR, 3-nitrobenzyl alcohol; IPTG, isopropyl β-D-thiogalactopyranoside.
UV (H₂O, 50 mM Tris-HCl, pH 7.5): λₘₐₓ (ε): 463 nm (5280).

Enzyme Assay—Flavin reductase activity was determined at room temperature from the decrease of the absorbance at 340 nm (ε₃₄₀ = 6.22 mM⁻¹ cm⁻¹) due to the oxidation of NADPH, using a Kontron Uvikon 930 spectrophotometer. Under standard conditions, the spectroscopic cuvette contained, in a final volume of 500 μl, 50 mM Tris-HCl, pH 7.5, and variable concentrations of NADPH, NADH or flavins. The reaction was initiated by adding 0.3 μg of enzyme except in the case of Fig. 3 where 0.5 μg was used. One unit of activity is defined as the amount catalyzing the oxidation of 1 nmol NADPH/min. The hydrophobic compounds lumichrome, alloxazine, and lumiflavins were dissolved in Me₂SO. For lumichrome and lumiflavin, stock solutions were prepared by a further 1:3 dilution with water (final Me₂SO concentration, 3.5 mM), and in the enzymatic assays the final Me₂SO concentration was 87 mM. For alloxazine, the Me₂SO concentration in the assay was 350 mM. Me₂SO concentration up to 350 mM had no measurable effect on Kₘ and Vₘₐₓ values.

Construction of Plasmid pFN3 That Overexpresses Fre—Plasmid pEE1011 (16) contains the coding part of the flavin reductase gene, a 0.8-kilobase CiaI–BglII fragment cloned into the EcoRI and BamHI sites of pUC18. In order to have an unique Sall site located into the fre gene, the Sall site of the polylinker site was removed as follows. Plasmid pEE1011 was cleaved with XbaI and PstI and then incubated with the Klenow enzyme in the presence of dNTP. After ethanol precipitation, the generated blunt ends were religated and transformed with the Klenow enzyme in the presence of dNTP. After ethanol precipitation, the generated blunt ends were religated and transformed into DH5α. The recombinant plasmids were analyzed with restriction enzymes. Plasmid pEE1014, which contains only one Sall site located into the fre gene, was chosen for further studies. Plasmid pEE1014 was cleaved with EcoRI and HindII and the 0.8-kilobase fragment containing the fre gene was ligated with plf J119EH (26), which was cleaved with EcoRI and HindII. The resulting vector was named pFN3. This construction allowed the expression of the fre gene under the control of the IPTG-inducible tac promoter.

Overexpression and Purification of Fre—E. coli K12 carrying a plasmid pFN3 plasmid was grown at 37°C in Luria-Bertani medium (9 × 500 ml of Luria-Bertani medium in a 3-liter Erlenmeyer flask) in the presence of 100 μg/ml ampicillin. Growth was monitored by following the absorbance at 600 nm. Induction of Fre recombinant protein was done by adding IPTG to a final concentration of 0.4 mM when the optical density of the solution was about 0.3. The cells were collected by centrifugation at the late exponential phase. Extraction of bacteria was performed by lysozyme digestion of the cell membrane and freeze-thawing cycles as described in (4). All of the following operations were carried out at 4°C. After ultracentrifugation at 45,000 rpm during 90 min in a Beckman 50 Ti rotor, the supernatant was used as soluble extract for further purification. Soluble extract (~30 ml, 1 g of protein) was treated with 30% (w/v) ammonium sulfate for 30 min and then centrifuged for 20 min at 14,000 × g. The supernatant was loaded onto a phenyl-Sepharose column (20 ml) previously equilibrated with 25 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 30% (w/v) ammonium sulfate (buffer A). Proteins were eluted at a flow rate of 1 ml/min⁻¹ with buffer A until the baseline was recovered. Then ammonium sulfate concentration in buffer A was changed to 5% (w/v), and a flow rate of 1 ml/min⁻¹ was maintained. When the absorption at 280 nm reached again the baseline, flavin reductase activity was eluted with 25 mM Tris-HCl pH 7.5, 10% (v/v) glycerol, 30% (w/v) ammonium sulfate (buffer B). Fraction containing flavin reductase activity were pooled and concentrated to 2 ml using a Diaflo cell equipped with a YM10 membrane. The concentrated enzyme solution was loaded onto a Superdex 75 column (120 ml) from Pharmacia previously equilibrated with buffer B. Proteins were eluted with buffer B at a flow rate of 0.8 ml/min⁻¹. Flavin reductase was eluted as a single well defined peak. Active fractions were pooled and stored at −80°C.

E. coli Growth Inhibition by Hydouracil and Lumichrome—E. coli K12 was used as wild type strain. E. coli LS1312 (frecan) was derived by ϕP1 transduction of K12 as already described (15). 100 ml of M9 medium, supplemented with 0.4% glucose and 140 μM lumichrome when necessary, was inoculated at 1% with a preculture collected in the early exponential phase (OD₆₀₀ = 0.1). Growth was monitored by following the absorbance at 650 nm, hydouracil (crystalline, freshly dissolved in culture media) was added at the beginning of the exponential phase at an absorbance of about 0.05.

RESULTS

Overexpression and Purification of Recombinant Fre—Plasmid pJ119EH, a pKK223–3 derivative, is a broad host range plasmid that contains a polylinker sequence flanked on one side by the inducible tac promoter and on the other side by two strong transcriptional terminators (26). The gene for the lac repressor, lacI', was also present in this plasmid, and as a consequence, the plasmid can be utilized in strains lacking the repressor.

E. coli K12 was transformed by plasmid pFN3, which contains the fre gene inserted at the polylinker site of plf J119EH, and overexpression was tested in Luria-Bertani medium in the presence of IPTG. Overexpression was maximal when the cells were in the late log phase. The flavin reductase specific activity of E. coli K12 soluble extracts was around 50 units/mg of protein. Typically, extracts from E. coli K12 carrying pFN3 gave values of 5,000–7,500 units/mg after IPTG induction, thus showing a 100–150-fold overexpression of the enzyme. On the basis of a specific activity of 130,000 units/mg for the pure flavin reductase, it can be estimated that the overexpressed Fre enzyme represents 5% of the total soluble proteins. Extracts from E. coli K12 (pFN3) obtained in the absence of IPTG gave activity values of 300 units/mg, confirming the good control of plf J119EH derivative plasmids by the inducer. In addition, during growth, no significant loss of pFN3 in the presence or absence of IPTG has been noticed.

A two-step purification protocol, with a phenyl-Sepharose chromatography followed by gel filtration on Superdex 75, has been developed. From 4.5-liter cultures of E. coli K12 (pFN3) and 1-g protein extracts, 15–20 mg of about 90–95% pure flavin reductase, as judged by SDS-polyacrylamide gel electrophoresis, was obtained. The yield of the purification was 45%. The specific activity of the purified flavin reductase was 120,000 units/mg.

Mechanistic Studies of the Flavin Reductase—The experiments described here have been done under aerobic conditions when reduced flavins were rapidly reoxidized by oxygen. The reaction was monitored by following the disappearance of the absorbance of NADPH at 340 nm. With this assay, we measured the dependence of the reaction on various flavins and on reduced pyridine nucleotides. For such a bisubstrate-biproduct reaction, mechanisms with respect to orders of substrate binding can be delineated by kinetic analysis. Double reciprocal plots of initial velocities versus substrate concentrations show intersecting patterns for the sequential mechanism and parallel patterns for the ping-pong mechanism (27). In Fig. 1, flavin reductase activity was measured as a function of NADPH concentration at several levels of riboflavin (Fig. 1A) and as a function of riboflavin concentration at several levels of NADPH (Fig. 1B). Initial velocities followed typical Michaelis-Menten kinetics because double reciprocal plots of the results show a series of lines. Moreover, a ping-pong mechanism can be excluded because the lines are intersecting each other at the same point to the left of the vertical axis. A sequential mechanism for flavin reductase is thus indicated. The equations representing the ordered sequential Bi Bi mechanism and the rapid equilibrium random sequential Bi Bi mechanism have identical functional dependence on both substrates. However the ordered mechanism may be experimentally distinguished from the random mechanism through inhibition studies (28).

When the enzyme activity was determined as a function of NADPH concentration in the absence or in the presence of three concentrations of AMP, a dead-end inhibitor, double reciprocal plots of values obtained revealed typical competitive inhibition kinetics (Fig. 2A). A rather large Kᵢ value (0.5 mM) for AMP can be determined from these lines. On the other hand, patterns of noncompetitive inhibition was observed with respect to riboflavin (Fig. 2B).

Furthermore, lumichrome is both a strong competitive inhibitor of riboflavin with a Ki value of 0.5 μM (Fig. 3A) and an uncompetitive inhibitor of NADPH (Fig. 3B). All these data
support the conclusion that the flavin reductase has an ordered mechanism with NADPH binding first (28).

The kinetic mechanism of product release has been determined by studying inhibition by products (29). When NADPH concentration was varied with a fixed concentration of riboflavin, inhibition by NADP<sup>1</sup> was found to be competitive with respect to NADPH with a $K_i$ value of 5 mM. When riboflavin was varied at a fixed NADPH concentration, NADP<sup>1</sup> appeared to inhibit noncompetitively (data not shown). This now suggests that the first product to be released is the reduced flavin, followed by NADP<sup>1</sup>.

Flavin Substrate Specificity of the Flavin Reductase—Scheme 2 shows the structures of the various flavin analogs studied. Table I shows the kinetic data obtained with the flavin reductase. The apparent $K_m$ values were determined in experiments where flavin reductase activity was measured as a function of substrate concentration under saturating concentrations of the other substrate. The $k_{cat}$ values were obtained from the experimental maximum velocity. In the presence of riboflavin, the $K_m$ values for NADPH and NADH are 30 and 9 mM, respectively. A more detailed mathematical treatment of the steady-state initial rate measurements, developed by Dalziel (30) in the case of dehydrogenases with an ordered mechanism, was also applied to Fre. It clearly showed that $K_m$ values for neither NADPH nor flavin have any simple physical significance as dissociation constants. However, some useful information can be obtained from this analysis. First, from the data of Fig. 1, treatment by the method of Dalziel yields $K_m$ values for NADPH (30 mM) and riboflavin (1.9 mM) similar to those reported in Table I, which had been determined by varying the concentration of the flavin at a fixed high concentration of NAD(P)H. Thus the $K_m$ values shown in Table I are approximately the same as those of the true kinetic constants. Second, the dissociation constant $K_{d}$ value for NADPH can be obtained from the data of Fig. 1. We found that the $K_{d}$ of NADPH is 61 mM, a value slightly different from the $K_m$ of NADPH of 30 mM. That the $K_{m}$ value of NADPH does not reflect the $K_{d}$ value of NADPH is obvious from the linear dependence of $K_m$ of NADPH with respect to $k_{cat}$ as described in (30). We verified

![Figure 1](https://example.com/fig1.png)  
**Fig. 1.** A, flavin reductase activity as a function of NADPH concentration in the presence of 1 (■), 2 (○), or 4 mM (●) riboflavin. B, flavin reductase activity as a function of riboflavin concentration in the presence of 24 (■), 36 (○), or 60 mM (●) NADPH. The results are presented as double reciprocal plots with straight lines determined by a linear regression program.

![Figure 2](https://example.com/fig2.png)  
**Fig. 2.** A, AMP as a competitive inhibitor for NADPH. The enzyme activity was assayed as a function of NADPH concentrations using 15 μM riboflavin in the absence (●) or in the presence of 200 (■), 400 (▲), or 600 μM (△) AMP. B, AMP as a noncompetitive inhibitor for riboflavin. The enzyme activity was assayed as a function of riboflavin concentrations using 180 μM NADPH in the absence (●) or in the presence of 0.5 (■), 1 (▲), or 2 mM (△) AMP.

![Figure 3](https://example.com/fig3.png)  
**Fig. 3.** A, lumarichrome as a competitive inhibitor for riboflavin. The enzyme activity was assayed as a function of riboflavin concentrations using 180 μM NADPH in the absence (●) or in the presence of 0.5 (■), 1 (▲), or 2 mM (△) lumarichrome. B, lumarichrome as a noncompetitive inhibitor for NADPH. The enzyme activity was assayed as a function of NADPH concentrations using 15 μM riboflavin in the absence (●) or in the presence of 1 (▲), 3 (○), or 5 mM (△) lumarichrome.
that for the various flavins studied here (riboflavin and lumiflavin) $k_{\text{cat}}/K_m$ of NADPH was a constant equal to the rate of binding of NADPH to the enzyme ($1 \times 10^6$ M$^{-1}$·min$^{-1}$).

On the other hand, the theory shows that it is impossible to determine the $K_m$ values for the flavin precisely. One has to rely on $K_m$ values to analyze how changes in flavin structure affects the flavin-protein interaction. In a first approximation, we may consider that large variations in $K_m$ values roughly reflect variations in flavin recognition by the enzyme when the substrates give $k_{\text{cat}}$ values of the same order of magnitude.

As previously reported (4), the $K_m$ values for riboflavin and FMN were in the 1–3 μM range with either NADPH or NADH. Riboflavin in both cases gave the highest $K_m$ value, but the differences between flavins were much greater with NADPH.

The presence of a terminal phosphate group on the ribityl chain, as in FMN, greatly altered the catalytic efficiency of the reaction. FAD gave no reaction with NADPH. Also the introduction of a H-phosphonate group led to a large decrease of the $k_{\text{cat}}$ value (compare compounds 4 and 6).

Lumiflavin, with a methyl group at N-10, was also a substrate with $K_m$ values similar to those of natural substrates and $k_{\text{cat}}$ larger than that of FMN. This now shows that the ribityl chain is not essential for recognition by the enzyme. Accordingly, ribitol has no inhibitory effect on the NADPH-rumiflavin reductase activity even at 100 mM concentration. Moreover, as shown above, lumichrome, which lacks the ribityl chain, is a very efficient competitive inhibitor with respect to riboflavin.

On the other hand, $k_{\text{cat}}$ but not $K_m$ can be modulated significantly by chemical modification of the sugar chain. Charge, discussed above, is not the only parameter, because a large difference in $k_{\text{cat}}$ is also observed between riboflavin and lumiflavin. In order to get deeper insight into such a modulation, we also tested a series of flavin derivatives in which the sugar moiety has been modified. Table I shows that the 2′-OH may play a role in the NADPH-dependent reaction because similar catalytic efficiency ($k_{\text{cat}}/K_m$) is found for riboflavin and compounds 3 and 5, whereas the reaction was 2- and 5-fold less catalytically efficient with compound 4 and lumiflavin, respectively.

Because binding of the flavin molecule seems to occur mainly through the isalloxazine ring, it is important to determine which sites of that ring are participating to the recognition of the molecule by the polypeptide chain. First, methyl groups at C-7 and C-8 seem to play an important role in the binding because compound 8, the lumiflavin derivative that lacks these methyls, is also a substrate but with a $K_m$ value 10-fold larger than that for lumiflavin. Furthermore, alloxazine, the lumichrome analog lacking the methyl groups, is also an inhibitor but with a $K_i$ value about 200-fold larger than that for lumichrome. The only compound in this study containing a modification at N-5 was riboflavin N-oxide (13d). The catalytic activity of the enzyme was not affected by the presence of the oxo group (data not shown).

Methylation at the N-3 of riboflavin (compound 7) greatly decreased the catalytic efficiency of the reaction, due to both a large increase of the $K_m$ value and a large decrease of the $k_{\text{cat}}$ value. Because N-3 plays only a limited role in flavin redox chemistry, with redox potentials insensitive to N-3 alkylation,

| Flavin derivative | Properties | $K_m$ (μM) | $k_{\text{cat}}$ (μM·min$^{-1}$) | $k_{\text{cat}}/K_m$ (μM) | $K_i$ (μM) |
|-------------------|------------|------------|-------------------------------|--------------------------|------------|
| Riboflavin        | Substrate  | 2.5        | 3144                          | 1257                     | 0.5        |
|                   |            | (1.3)      | (1834)                        | (1410)                   | 96         |
| FMN               | Substrate  | 2.2        | 161                           | 73                       |            |
|                   |            | (1.5)      | (681)                         | (454)                    |            |
| Lumichrome        | Inhibitor competitive/riboflavin |            |                               |                          |            |
| Alloxazine        | Inhibitor competitive/riboflavin |            |                               |                          |            |
| Ribitol           | No inhibition up to 100 mM |            |                               |                          |            |

$^a$ $k_{\text{cat}}$ is expressed in μM·min$^{-1}$.

$^b$ $k_{\text{cat}}/K_m$ is expressed in 10$^6$ min$^{-1}$·μM$^{-1}$. 

**Scheme 2. Structure of the riboflavin derivatives and synthesis intermediates.** Compounds 11, 12, and 13 were prepared with different R$_2$ chains referred to as a for R$_2$ = CH$_2$CH$_2$OH, b for R$_2$ = (CH$_2$)$_2$CH$_2$OH, and c for R$_2$ = CH$_2$CH$_2$OH-CH$_2$OH. Compound 13d was with R$_2$ = 1'-deoxyribitol.
it appears that this site may be involved in flavin binding (31).

Finally, compounds 12, which contain both the dimethylphenyl and pyrimidine moieties, were totally devoid of inhibitory properties (data not reported), showing that the binding site of the enzyme has a specific requirement for the whole isoalloxazine ring.

Inhibition of the Reactivation of Ribonucleotide Reductase by Lumichrome in Vivo—The small subunit of ribonucleotide reductase, named protein R2, contains a tyrosyl radical in its active form. Inactivation of the enzyme and thus inhibition of DNA synthesis can be achieved by hydroxyurea, which scavenges the radical and generates the inactive form, named metR2. Such a reaction is the basis of the utilization of hydroxyurea as an antiproliferative agent in clinics (5). We have previously shown that the flavin reductase in E. coli is involved in the repair of the ribonucleotide reductase activity by regenerating the radical and thus transforming metR2 to R2. The flavin reductase thus protects E. coli from radical scavengers such as hydroxyurea (15). The present discovery that lumichrome was a good inhibitor of the E. coli flavin reductase led us to investigate the potential of a combination hydroxyurea/lumichrome for inhibition of bacterial growth. The data are shown in Fig. 4.

When E. coli K12 cells were grown aerobically in M9 medium, the addition of 40 mM hydroxyurea at an OD650 of about 0.05 resulted, as expected, in a significant decrease of the growth rate. Lumichrome, instead, had no effect. When now a combination of 40 mM hydroxyurea and 140 μM lumichrome was added to the culture medium, bacteria stopped growing totally, indicating a strong and remarkable synergic effect of the combination.

Similar behavior was observed when the flavin reductase was inactivated genetically. Growth of an E. coli mutant strain, LS1312, lacking an active fre gene was fully inhibited by addition of 40 mM hydroxyurea alone, confirming the function of the flavin reductase during repair of hydroxyurea-treated E. coli cells. As far as growth inhibition by hydroxyurea is concerned, addition of lumichrome or inactivation of the fre gene gave similar phenotypes.

**DISCUSSION**

In order to study the structure and the mechanism of the NAD(P)H:flavin oxidoreductase from E. coli, an overproducing strain of E. coli was obtained. The transformation of E. coli K12 by plasmid pFN3, which contains the fre gene under the control of the tac promoter, led, after IPTG induction, to a 100–150-fold overexpression of the enzyme. Such an overexpression allowed purification of the soluble enzyme by two chromatographic steps, phenyl-Sepharose and Superdex 75. Crystallization of such preparations is presently under investigation.

The NAD(P)H:flavin oxidoreductase from E. coli is the prototype of the class of flavin reductases, which do not contain any light-absorbing (flavins, metals) prosthetic group for mediating the electron transfer from reduced pyridine nucleotide to free oxidized flavin. The kinetic analysis of the enzymatic reaction demonstrated that the flavin reductase has an ordered mechanism (Scheme 3). The catalysis by the 26-kDa polypeptide chain is thus achieved by providing a site where both substrates bind and interact: NADPH binds first to the active site, followed by the flavin. After electron transfer, the first product to be released is the reduced flavin, followed by

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**FIG. 4.** Inhibition of cell growth by lumichrome and hydroxyurea. Growth of E. coli K12 or LS1312 under standard conditions (●) or in the presence of 140 μM lumichrome (○), 40 mM hydroxyurea (▲), or 40 mM hydroxyurea and 140 μM lumichrome (◆).

**SCHEME 3.** Proposed reaction mechanism of the flavin reductase Fre.
NADPH. This is in full agreement with AMP and NADP\(^+\) being competitive inhibitors with respect to NADPH and noncompetitive with respect to flavin and lumichrome being a competitive inhibitor with respect to flavin and noncompetitive with respect to NADPH. Similar results were obtained in the case of flavin reductase D, the NADH-dependent flavin reductase from V. harveyi (32).

The remarkable efficiency of lumichrome as an inhibitor of the flavin reductase in vitro (K\(_{i1}\) = 500 nM) led us to test whether it may also affect the activity under in vivo conditions. As a matter of fact, lumichrome greatly potentiated the inhibitory effect of hydroxyurea, a specific inhibitor of ribonucleotide reductase, on the growth of E. coli K12 cells (33). This is a very interesting observation because (i) it is in good agreement with the observation that in bacteria, flavin reductase plays a crucial role in the activation of hydroxyurea-inactivated ribonucleotide reductase and (ii) it shows for the first time that the combination of an inhibitor of ribonucleotide reductase such as hydroxyurea and an inhibitor of flavin reductase might have potential applications for inhibition of DNA synthesis and for general antiproliferative activity. Whether such a strategy could be applied not only to microorganisms but also to human beings, for example for cancer treatment, is of course just speculative. It is important also to note that very recently hydroxyurea has received renewed attention in the context of AIDS research because it was found to greatly potentiate the anti-HIV effects of nucleoside analogs such as 2′,3′-dideoxyninosine (34).

The riboflavin substrate is composed of two distinct regions, a highly hydrophobic isoalloxazine ring and a ribityl side chain linked at N-10. The results reported in this study show that the binding to the polypeptide chain occurs mainly through the isoalloxazine ring as a whole. This is clear from the following observations: (i) lumichrome, an analog of isoalloxazine with no sugar chain, is a very good inhibitor with a low K\(_{i}\) (0.5 \(\mu\)M), indicating a strong binding to the enzyme, while ribitol has no inhibitory properties; (ii) the rigidity of the ring seems to be an important parameter, because compounds 12 have no inhibitory effects; (iii) similar K\(_{i}\) values were found for riboflavin, lumiflavin, and for flavin analogs with various side chains. The same K\(_{i}\) values were found for FMN, FAD, and compound 6 in spite of the presence of a negatively charged group and of the important size of the side chain. In addition, the methyl groups at position 7 and 8 (compare the K\(_{i}\) for lumichrome and alloluxazine and the K\(_{i}\) values for lumiflavins and compound 8) and nitrogen at position 3 (compare the K\(_{i}\) values for riboflavin and compound 7) play an important role in the binding of the flavin ring.

In the absence of a three-dimensional structure for the enzyme and for a substrate-enzyme complex, it is just possible to use our data as a basis for predicting some structural characteristics of the flavin reductase involved in flavin recognition. The following analysis was based on the refined structures of flavoproteins. In flavodoxin (35) and ferredoxin-NADP\(^+\) reductase (36), possessing FMN and FAD, respectively, as an integral component of the protein, aromatic residues (tryptophan or tyrosine) are involved in the recognition of the isoalloxazine ring through \(\pi\)-orbital overlaps. This seems to be a general strategy that may operate in the case of flavin reductase as well. On the other hand, in flavoproteins, extensive interactions also exist between the protein and the ribityl phosphate or ribityl phosphate-AMP and contribute to the overall affinity of the apoprotein for the cofactor significantly. These interactions seem to be very weak in the case of the flavin reductase, in agreement with the low specificity of the enzyme as well as its weak affinity for flavin substrates.

On the other hand, as shown in Table I, some of the hydroxyl groups of the ribityl chain seem to slightly contribute to decreasing the activation barrier for catalysis. Actually, in the presence of NADPH, the k\(_{cat}\) value for lumiflavin is about 5-fold lower than that for riboflavin. The magnitude of the effect of the ribityl group is most conveniently expressed as a decrease in the free energy of activation, calculated from the corresponding k\(_{cat}\) values according to 3.5  =  \(\Delta G^\circ\) = \(-RT\ln k_{cat(r)}/k_{cat(l)}\) (lumiflavin)), where k\(_{cat(r)}\) (ribo) and k\(_{cat(l)}\) (lumiflavin) refer to riboflavin and lumiflavins, respectively. The decrease in the free energy of catalysis is about 0.9 kcal/mol, indicating a limited but nevertheless significant role of the sugar chain in enzyme catalysis. From the substrates tested here, it is possible to identify the important hydroxyl groups for this effect. The 4′- and 5′-OH do not contribute at all because compound 5 had the same k\(_{cat}\) value as riboflavin. On the other hand, 2′-OH and to a lesser extent 3′-OH may be involved in decreasing the activation barrier of the reaction (compounds 3 and 4).

Catalysis seems to be also controlled by the charge of the side chain. Actually with NADPH, which contains a pyrophosphate and a phosphate group, as a reducing agent, the k\(_{cat}\) value for FMN, which contains one phosphate group, is 20-fold smaller than that for the neutral riboflavin, and moreover the enzyme is unable to catalyze the reduction of FAD, which contains a pyrophosphate group. Furthermore, weaker discrimination is obtained with NADH (riboflavin:FMN:FAD = 1:0.37:0.37), which has one phosphate group less than NADPH. The negative effect of a charge on the flavin substrate is also seen from the low activity of compound 6.

In addition, it is interesting to note that, as an FMN reductase, Fre is more specific for NADH. This further supports the similarity to flavin reductase D, one of the flavin reductases from V. harveyi, which has been described, with FMN as the electron acceptor, to be specific for NADH (13).

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