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Further Computational Studies on the Conformation of 1,5-Dihydrolumiflavin

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ABSTRACT

The optimized geometry of 1,5-dihydrolumiflavin has been calculated using density functional theory (DFT). Reduced lumiflavin was found to be bent along the N5-N10 axis, 25° from planarity, which is nearly the same as previously reported restricted Hartree–Fock (RHF) calculations, which predict a bending angle of 27°. The major difference in the DFT calculation is that the N10 methyl group adopts a more pseudoequatorial disposition and is only bent 13° above the plane of the isoalloxazine ring system as opposed to 59° in the RHF calculations. These computational results are compared with x-ray crystal structures of flavin models and flavoproteins. DFT calculation of 1,5-dihydroisoalloxazine resulted in a more modestly bent geometry of 17°. This indicates that both electronic and steric interactions of the N10 methyl group of reduced lumiflavin contribute to the bent geometry. Antioxid. Redox Signal. 5, 737–746.

INTRODUCTION

IT IS REASONABLY well accepted that the preferred conformation of the isoalloxazine ring system of flavin coenzyme in the oxidized and semiquinone states is planar (8). The conformation of fully reduced flavin, however, has been debated. Early light absorption studies concluded that fully reduced, free flavin was nonplanar (2). It has been suggested that the central pyrazine ring of 1,5-dihydroflavins contains eight π-electrons and therefore is a Hückel antiaromatic system. To alleviate this antiaromatic destabilization, flavin hydroquinone adopts a bent or "butterfly" conformation, folding along the N5-N10 axis. However, Hückel's rule was developed for conjugated monocyclic compounds, and its application to a portion of a conjugated polycyclic system is tenuous at best.

X-ray crystallographic analysis of flavin models **1**–**7** (Fig. 1), which are formally in a reduced state, provided strong support for the "butterfly" geometry of flavin hydroquinone (9, 11, 21, 22, 24, 27, 29, 30). For flavin models **1**–**5,** the bending angle is reported to be between 30° and 36° (Fig. 2), whereas **6** and **7** showed much less dramatic bending of 9° and 13°, respectively (Table 1). The sensitivity of 1,5-dihydroflavins toward facile air oxidation required that the x-ray analysis be performed on flavin analogues substituted at the N1 and/or N5 positions. It has been argued that the bent geometry observed in these crystal structures might be a result of the increased steric demands of these substituents (10). In contrast to the crystallographic analyses, NMR studies on reduced free flavins have been interpreted as showing a planar or slightly bent geometry (17, 18).

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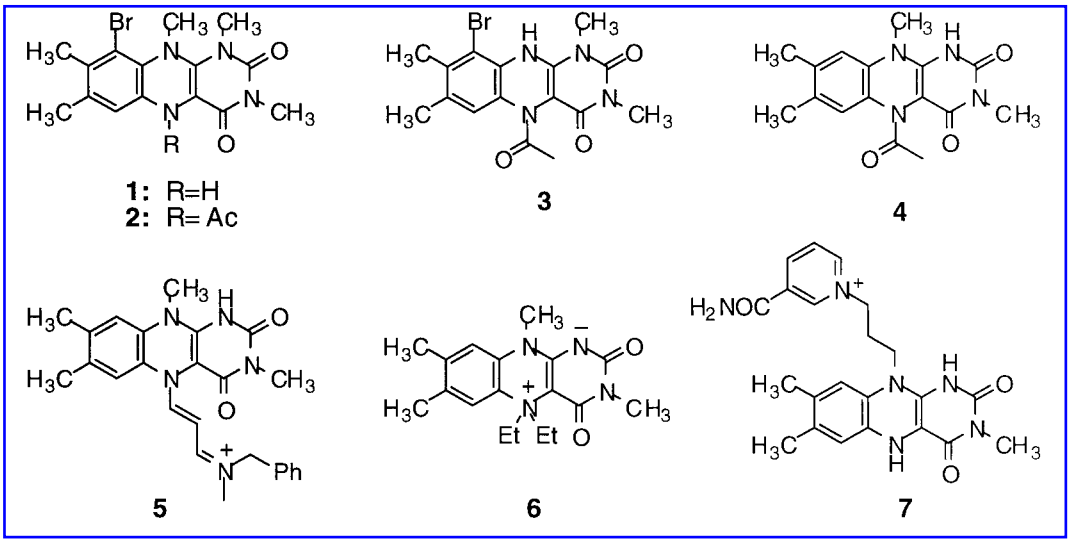

FIG. 1. Reduced flavin models previously studied by x-ray crystallography. Ac, acetyl; Et, ethyl; Ph, phenyl.

The dramatic difference in conformation between fully reduced flavin from the other oxidation states led to the proposal that control of conformation could be one mechanism by which the protein environment influences the redox and catalytic properties of the flavin coenzyme (14, 28). If the apoenzyme was able to deform the preferred planar structure of the oxidized flavin coenzyme so that it more closely resembles the bent reduced structure, then the cofactor would become a better electron acceptor. Conversely, forcing 1,5-dihydroflavin to adopt a higher energy planar conformation would result in a better electron donor. This hypothesis is supported by protein crystallography of flavoproteins in various oxidation states. The conformation of the flavin cofactor of 190 flavoproteins in the Protein Data Bank (PDB) was recently surveyed, from which 56 unique conformations of the cofactor were identified (46 oxidized, two semiquinones, eight dihydroflavins) (12). Most of the oxidized flavoproteins possessed isoalloxazine rings within 6° of planarity. Several oxidized cofactors, however, showed significant bending along the N5-N10 axis and more closely resembled the predicted structure of the reduced cofactors. These include trimethylamine dehydrogenase (27°), pyruvate oxidase (18°), and NADH oxidase (18°) among others. The isoalloxazine conformation of the two flavoproteins in the semiquinone state were relatively planar with bending angles of 7° and 4° for flavocytochrome b2 and flavodoxin, respectively. Of the eight crystal structures of fully reduced flavoproteins, only three showed significantly bent conformations, old yellow enzyme (DYE) (18°), cholesterol oxidase (ChO) (15°), and thioredoxin reductase (TrxR) (34°). The others are within 7° of planarity.

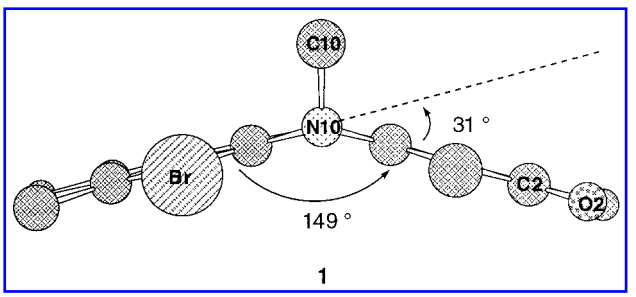

FIG. 2. Bent geometry of flavin model 1 determined by x-ray analysis.

Computational studies utilizing semiempirical (1, 5–7), *ab initio* (16, 19, 23, 26, 31, 32), and density functional methods (15, 25) have been used extensively to aid in predicting the structure and properties of flavin coenzyme (Table 2). In agreement with experimental evidence, computational studies universally predict a planar geometry for the oxidized cofactor, and nearly all predict that the semiquinone is also planar. An extensive computational study by Zheng and Ornstein examined all three oxida-

Table 1. Conformation of Flavin Models and Protein Bound Cofactors

Flavin	*CSD (PDB) code*	*Bending angle*[a]	*RMSD benzene*	*RMSD pyrimidine*	*Ref.*
1	abmial	150° (149)°	0.031	0.019	19
2	bhmalx10	144° (145°)	0.011	0.050	28
3a	abmhal	151° (151°)	0.044	0.066	11
b		148° (148°)	0.034	0.099	
4	athalx	(148°)	NA	NA	21
5	miphix	150°	0.046	0.075	26
6	emhial	171° (171°)	0.031	0.019	29
7 (red)	cpmhia10	166° (167°)	0.046	0.031	23
7 (ox)	cpmial10	180° (179°)	0.016	0.027	23
3-Methyl lumiflavin (ox)	mlumif	179°	0.016	0.027	22
OYE (red)	(1oyc)	164° (168°)	0.051	0.087	3
OYE (ox)	(1oya)	174° (173°)	0.030	0.072	3
ChO (red)	(1coy)	166° (165°)	0.060	0.062	13
ChO (ox)	(3cox)	170° (168°)	0.051	0.068	13
TrxR (red)	(1c10)	148° (146°)	0.043	0.025	12
TrxR (ox)	(1tde)	178° (176°)	0.033	0.025	12

CSD, Cambridge Structural Database; PDB, Protein Data Bank; RMSD, root mean square deviation.
[a]Previously reported bending angles are in parentheses.

tion states of lumiflavin in various protonation states of each, using the restricted Hartree–Fock (RHF) method for the oxidized and fully reduced state and unrestricted Hartree–Fock for the semiquinone (32). The geometries were optimized using a 6-31G* basis set for neutral and cationic species and 6-31+G* for anions. For neutral 1,5-dihydrolumiflavin, the optimized geometry was bent 27° from planarity along the N5-N10 axis. This bending of the reduced isoalloxazine rings is smaller than that found by x-ray analysis of flavin models **1–5.** Interestingly, the optimized geometry of the N1-deprotonated lumiflavin hydroquinone anion was predicted to be planar. This important finding may explain the near-planar geometry observed for the reduced cofactor in a number of flavoproteins as well as for model **6.**

We report here the results of a computational study in which the optimized geometry of 1,5-dihydrolumiflavin (**9**) was calculated using density functional theory (DFT) methods (Fig. 3). The optimized structure of **9** differs significantly from the previously report RHF/6-31G* optimized structure. In addition, we also examined the geometry of 1,5-dihydroisoalloxazine (**11**) and lumiflavin hydroquinone anion (**10**). The N5-formyl-isoalloxazines (**12** and **13**)

Table 2. Calculated Conformation of Flavin Models

Flavin	*Theory*	*Bending angle*[a]	*RMSD benzene*	*RMSD pyrimidine*
8a	RHF/6-31G*	180°	<0.01	<0.01
	B3LYP/6-31G*	180°	<0.01	<0.01
9	RHF/6-31G*	153° (153°)	0.025	0.011
Conformation 1	B3LYP/6-31G*	155°	0.020	0.022
Conformation 2	B3LYP/6-31G*	157°	0.022	0.020
10	RHF/6-31G+*	180° (180°)	<0.01	<0.01
11	B3LYP/6-31G*	163° (165°)	0.016	0.022
12	B3LYP/6-31G*	148°	0.019	0.035
13	B3LYP/6-31G*	153°	0.022	0.032

RMSD, root mean square deviation.
[a]Previously reported bending angles are in parentheses.

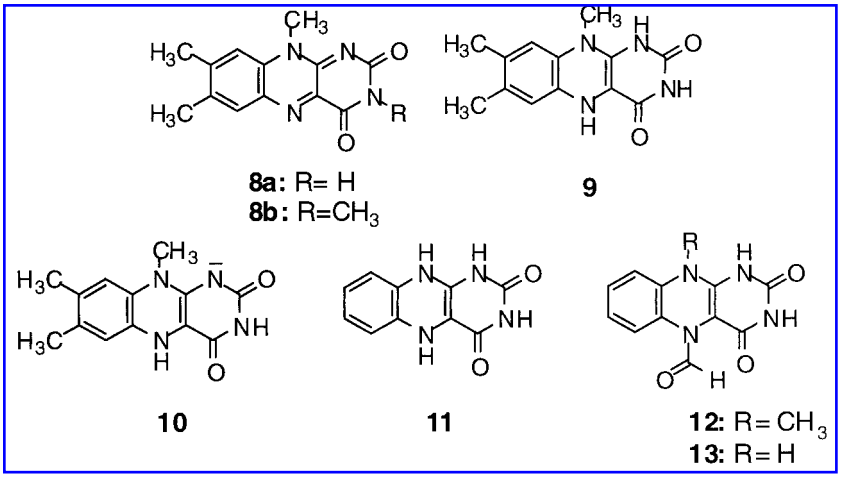

FIG. 3. Flavin models studied by computational methods.

were examined to determine the influence of the N5 acetyl group on the geometry of flavin models such as **1–4** (Fig. 1).

MATERIALS AND METHODS

The optimized geometries of **8a** and **9–11** were calculated using the B3LYP/6-31G* hybrid density functional method as implemented in GAUSSIAN 98W (revision A.9, Gaussian, Inc.) for Windows (4). Flavins **12** and **13** were calculated using a B3LYP/6-31G basis set. Calculations were run on a Gateway 2000 GP-7 850 Pentium III computer running Windows 98. Results were visualized with Chem-3D (v. 5.0, CambridgeSoft, Inc.) and GaussView (Gaussian, Inc.). Bending angles were determined with CrystalMaker (v. 4.03, CrystalMaker Software). This was done by calculating the angle between the intersecting planes fit to the six atoms of the benzene ring, plus the N5 and N10 atoms with the best fit plane comprised of the six ring atoms of the pyrimidine ring plus the N5 and N10 atoms. The best planes were calculated using a least-squares fit as implemented in CrystalMaker.

The RHF/6-31G* optimized geometry of **9,** as generously provided by Zheng and Ornstein, was used as a starting geometry for the DFT optimization (32). This input file was then modified to obtain the starting geometries for **10** and **11.** The atomic coordinates from the x-ray analysis of **2** were used as a starting geometry of **12** and **13.**

RESULTS

The geometries of **9** and **11** were optimized using the B3LYP/6-31G* method as implemented in GAUSSIAN 98, revision A.9 (4). In addition, the geometry of the oxidized form of lumiflavin (**8a**) was computed. The bending angle along the N5-N10 axis (see Fig. 2) was determined using the CrystalMaker software package as described in Materials and Methods. Our results are tabulated in Table 2. These results should be compared with the bending angles of flavin models **1–7** obtained from crystal structure data and the cofactors for OYE, ChO, and TrxR obtained from the PDB (Table 1). As the bending angles in the present study were determined in a slightly different manner from previous studies, the previously reported bending angles are also given. In general, the bending angles reported here are in good agreement with those previously reported.

The RHF optimized geometry of 1,5-dihydrolumiflavin (**9**) as determined by Zheng and Ornstein was reproduced (32). Optimization using the DFT B3LYP/6-31G* method gave a significantly different result in that two conformations of reduced lumiflavin were found, which differed by 0.55 kcal/mol. The higher energy conformation was nearly identical to

the Zheng and Ornstein structure. The bending angles along the N5-N10 axis for both DFT optimized conformations were similar to that obtained from the RHF calculations. In the favored conformation, the N10 methyl group adopted a more pseudoequatorial orientation and was the most significant structural difference. Optimization of **11** with a B3LYP/6-31G* basis set also gave a bent geometry, although the bending angle of 17° is smaller than that found for **9.** In contrast to the RHF results, DFT calculations indicate that lumiflavin hydroquinone anion is not planar.

Many of the x-ray analyses of flavin models (**1–4**) possessed an N5 acetyl group. To examine the influence of this acetyl group, the B3LYP/6-31G optimized structures of **12** and **13** were determined. To expedite the calculation, an N5 formyl group was used and the C7 and C8 methyl groups were omitted. The optimized structure of **12** was slightly more bent (29° from planarity) than **9** and agreed reasonably well with the crystal structures of **1–4.**

DISCUSSION

Like previous computational studies, the DFT method predicts 1,5-dihydrolumiflavin (**9**) to be bent along the N5-N10 axis. In the present study, two conformations of **9** were found that differed by 0.55 kcal/mol. The lower energy conformation is significantly different from that predicted by other methods (32). DFT and RHF calculations predict nearly identical bending angles along the N5-N10 axis, 155° versus 153°, respectively (Figs. 4 and 5). The more pronounced geometric difference is the disposition of the N10 methyl group. Using the N5-N10-C10 angle as a reference, the DFT method predicts an angle of 167° as compared with 115° for the RHF calculation. The N10 methyl group is displaced from the plane of the isoalloxazine ring system to a much lesser extent in the DFT calculations, permitting the N10 nonbonding electron pair to be nearly fully conjugated with the other π-electrons of the dihydroisoalloxazine (Fig. 5). The optimized geometry from RHF studies would predict a more pyramidal N10 and very little interaction of the electron pair with the rest of the π-system (Fig. 4).

The second conformation of **9** was slightly higher in energy and is nearly identical to the RHF optimized structure found by Zheng and Ornstein (32). The different conformations arise from a simple 60° rotation about the N10-C10 bond. In the lower energy structure, one of the C10 hydrogens bisect hydrogen atoms on C9 and N1, allowing for the flatter conformations (Fig. 5). In the second conformation, two of the C10 hydrogen atoms are somewhat eclipsed with the N1 and C9 hydrogens, resulting in a more pseudoaxial disposition of the C10 methyl group. In this conformation, the overlap of the nonbonding pair of electrons on N10 with the rest of the π-system is greatly reduced. These two conformations could be interconverted. The C10 methyl group of the optimized lower energy conformation was rotated 60° so that the eclipsing interactions were present. Optimization starting from this geometry gave

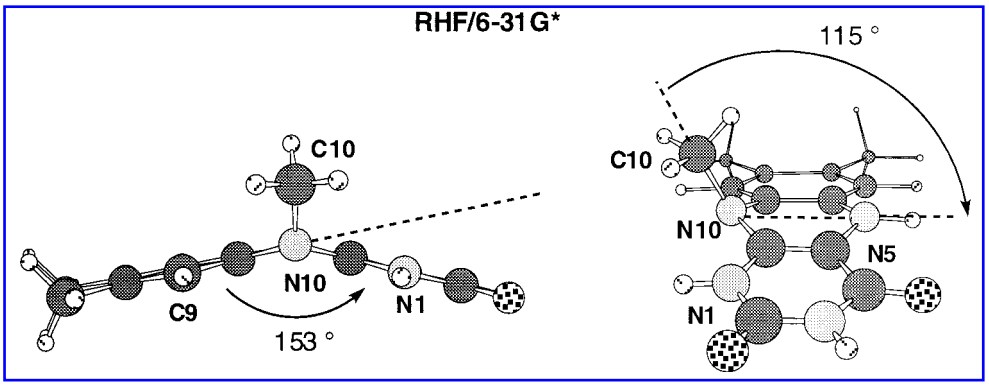

FIG. 4. RHF/6-31G* optimized geometry of 1,5-dihydrolumiflavin.

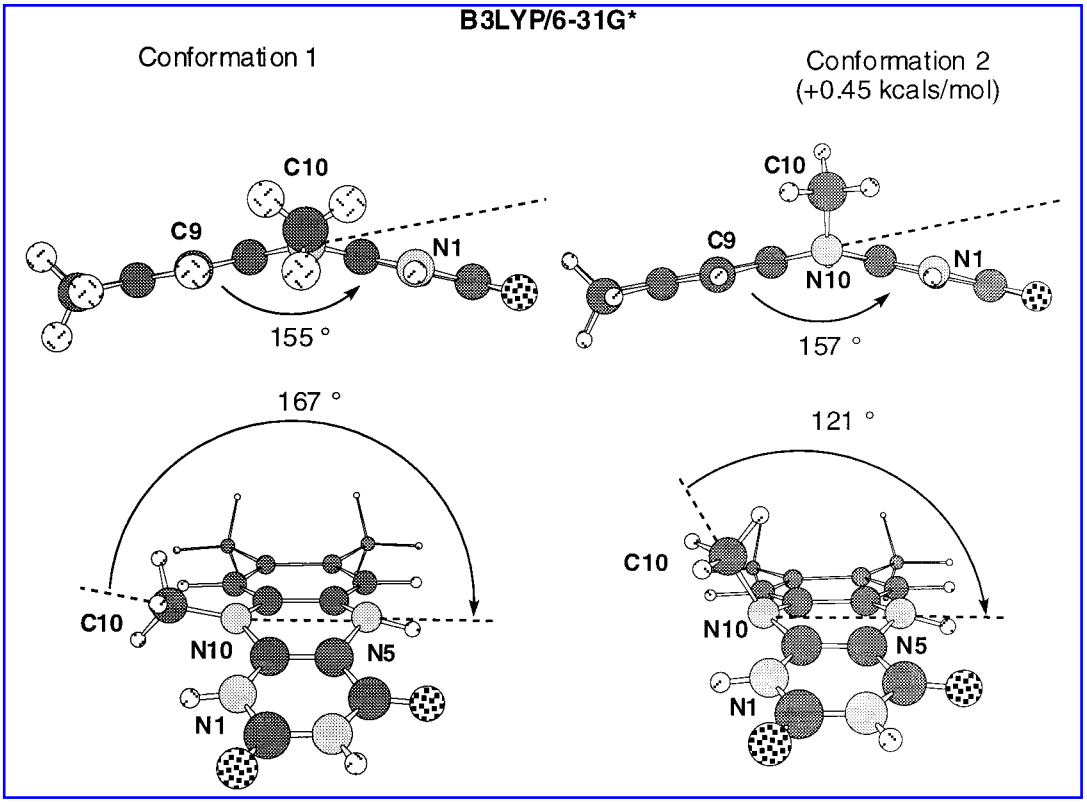

FIG. 5. B3LYP/6-31G* optimized geometries of 1,5-dihydrolumiflavin.

the higher energy structure. Alternatively, optimization of the higher energy conformation, after the C10 methyl group was rotated 60° to alleviate the eclipsing interactions, gave the lower energy geometry. When the RHF/6-31G* calculations were repeated using conformation 1 as a starting geometry, the conformation found by Zheng and Ornstein was obtained (32).

Previously, the significance of the bent geometry of **2,** observed by x-ray crystallography, was questioned because of large steric crowding of C9 bromo, N10 methyl, and N1 methyl groups (10). These steric interactions are partially relieved when a bent conformation is adopted. However, the x-ray crystal structures of models **3** and **4** showed conformations similar to that of **2,** indicating that steric crowding was not a major consideration. Meyer has examined the structure of 1,5-dihydroalloxazine (**10**) as well as the N1-deprotonated anion and N5-protonated cation at the B3LYP/6-31G* level. It was reported that all three were folded along the N5–N10 axis by 15°, 12°, and 21°, respectively (15). The computational results reported here indicate that the steric interactions of the N10 substituent contribute significantly to the overall geometry of fully reduced, free flavins and account for the flatter geometry of 1,5-dihydroisoalloxazine (**11**), which lacks an N10 substituent.

The disposition of the N10 substituent, as it relates to the conformation of reduced flavins, has not been previously addressed. Upon reduction of free flavins, this substituent adopts a pseudoaxial orientation, whereas in the oxidized form it is in the plane of the isoalloxazine ring (pseudoequatorial). For the biologically active cofactor, the N10 ribityl group is the main binding domain for the protein–cofactor complex and, as such, this group does not have the same degrees of conformational freedom as in free flavin. When crystal structures of flavoproteins are examined, it is found that this substituent does not change orientations as in free flavins. An example is OYE shown in Fig. 6. In the bent reduced form of OYE, the position of the C1′ atom is relatively unchanged from the oxidized state, as measured by the N5-N10-C1′ angle as well as the dihedral angle of C1′ to N1

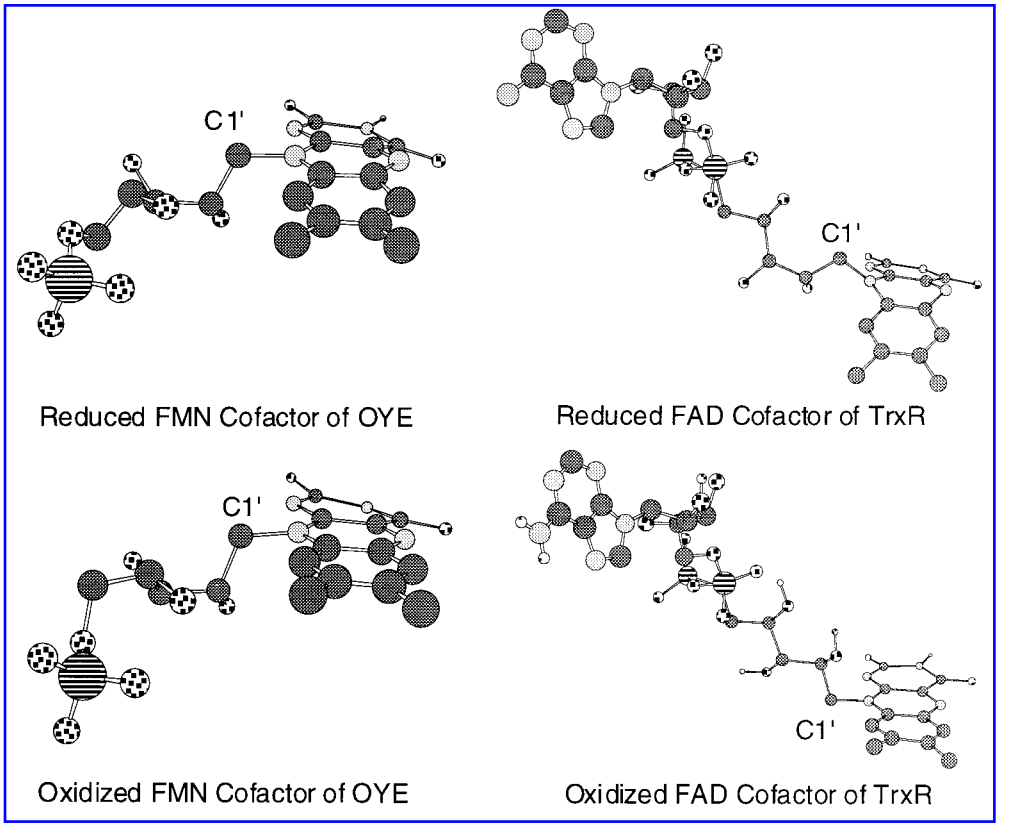

FIG. 6. Geometry of the oxidized and reduced cofactors of OYE and TrxR. FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

or C9 (Table 3). This is true even in distorted geometries of oxidized flavin cofactors such as trimethylamine dehydrogenase. One exception is the fully reduced form of TrxR (Fig. 6), where the C1′ atom is significantly shifted, ~16° above the plane of the isoalloxazine ring (12). It was reasoned that a bent geometry with the N10 substituent in a pseudoequatorial position might also be a local minimum energy conformation as in the structure of reduced OYE. As hydrogens are not explicitly found in protein structures, a pyramidal N5 atom was assumed and the N5 proton was placed in a pseudoaxial position above the flavin ring system. Geometry optimization gave the identical structure as earlier. This suggests that the protein environment enforces the observed pseudoequatorial orientation of the ribityl group in bent cofactors, with the exception of reduced TrxR. To explore further the relative energy of a bent 1,5-dihydrolumiflavin with a pseudoequatorial N10 methyl, calculations were performed in which the dihedral angle from C9 to C10 was fixed at 0° while allowing the rest of the molecule to optimize. The geometry was found to be only 0.35 kcal/mol higher in energy than the lowest energy conformation of **9.** The bending angle of this structure was 25° from planarity.

As mentioned, Zheng and Ornstein reported that the RHF/6-31G* optimized geometry of the lumiflavin hydroquinone anion was planar (32). With the pK_a of the N1 position of dihydroflavins at ~6.5, this result may explain the planar geometry of a number of reduced flavin cofactors observed in protein crystal structures. The cofactors of many fully reduced flavoproteins have been determined to be in the deprotonated state and would be expected to be planar given these results. The relatively planar geometry of flavin model **5** can also be attributed to the fact that it deprotonated. In contrast to these results, Meyer reported that B3LYP/6-31G* calculations of the N1-deprotonated reduced isoalloxazine gave an optimized geometry that was bent along the N5-N10 axis by ~13° (15). A preliminary calculation of the reduced lumiflavin anion (**10**) was performed

Table 3. Orientation of the N10 Substituent

Flavin	*N5-N10-C10 angle*	*C9-C9a-N10-C10 dihedral angle*	*N1-C10a-N10-C10 dihedral angle*
1	111°	80°	81°
2	115°	76°	78°
5	168°	13°	14°
6	176°	6°	4°
7 (red)	173°	4°	5°
(ox)	176°	5°	4°
8b	178°	1°	3°
OYE (red)	178°	5°	4°
OYE (ox)	172°	7°	8°
ChO (red)	176°	15°	7°
ChO (ox)	178°	10°	3°
TrxR (red)	164°	46°	42°
TrxR (ox)	178°	4°	0°
8a			
B3LYP/6-31G*	180°	0°	0°
9			
RHF/6-31G*	115°	75°	74°
B3LYP/6-31G*			
Conformation 1	167°	14°	15°
Conformation 2	121°	67°	68°
10			
RHF/6-31+G*	178°	0°	0°

using the B3LYP/6-31G* basis set. A planar geometry was obtained from a planar starting geometry. A frequency calculation of this structure gave a single negative vibrational frequency, indicating that the planar geometry of the reduced flavin anion was a transition structure. The negative vibration corresponded to the bending motions along the N5-N10 axis, suggesting that the minimum energy conformation is bent along this axis. Although more work is necessary to determine the preferred structure of **10,** these preliminary results are consistent with the bent geometry of the reduced flavin anion, but the degree of nonplanarity has not been precisely determined at this time.

A number of previous computational studies on flavin coenzyme examined the energetic cost of perturbing the optimized geometry. For instance, it required <6 kcal/mol to planarize 1,5-dihydrolumiflavin from it preferred bent geometry. Bending oxidized lumiflavin along the N5-N10 axis, however, was energetically more costly. This led to the conclusion that oxidized flavin was "stiffer" than its fully reduced counterpart (12). This conclusion is in accord with a recent analysis of the normal vibrational modes of various oxidation states of isoalloxazine calculated at the RHF/6-31G* level (19). For reduced isoalloxazine and the corresponding semiquinone, the lowest energy vibrational mode was a "butterfly" bending motion along the N5-N10 axis. For 1,5-dihydroisoalloxazine, the total Hartree–Fock energy remained constant as the bending angle was changed ±10° from its equilibrium position of 16°. Flattening the fully reduced form resulted in a conformation only 2 kcal/mol higher in energy. The en-

ergy dramatically increased if the structure was bent >28°. Interestingly, the "butterfly" bending motion is not a normal vibration mode for the fully oxidized form, which probably accounts for the greater energetic cost of enforcing this conformation. The lowest vibrational mode of oxidized isoalloxazine is a twist along the long axis of the tricyclic system. This twisted geometry is commonly observed in oxidized flavoproteins. This analysis of the vibrational modes is consistent with those obtained from the B3LYP/6-31G* optimized geometries.

In conclusion, the conformation of 1,5-dihydroflavin was examined using density functional methods. The optimized geometry of fully reduced flavin was found to be bent along the N5-N10 axis as observed in previous computational studies. However, the disposition of the N10 methyl group allows for conjugation of the N10 lone pair with the π-system of reduced isoalloxazines. This appears to be the major difference with the RHF calculations in which conjugation is disrupted by the pseudoaxial orientation of this substituent. The DFT results show a bending angle of 25°, which is larger than that observed in some reduced flavoproteins, such as OYE (18°) and ChO (15°). DFT calculations of 5-formyl-1,5-dihydroisoalloxazine (**12** and **13**) also predicted significantly bent geometries, whereas the optimized geometry of 1,5-dihydroisoalloxazine (**11**) was only modestly bent. These calculations indicate that both electronic factors and steric interactions contribute significantly to the bent geometry of fully reduced, free flavins.

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ABBREVIATIONS

ChO, cholesterol oxidase; DFT, density functional theory; OYE, old yellow enzyme; PDB, Protein Data Bank; RHF, restricted Hartree–Fock; TrxR, thioredoxin reductase.

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