remains in the gauche–gauche conformation. It can be proposed that Li^+ bridges the carboxylate group of one PS molecule with the phosphate group of a neighboring molecule. Such a situation requires that both the carboxylate and phosphate groups lie in a plane approximately parallel to the bilayer plane. Further experiments to clarify this point are in progress.

In comparing the effects of Li^+ and Ca^{2+} on PS, we note that monolayer studies (Demel et al., 1987) have shown that Li^+ has little effect on the force–area curves of different phosphatidylserines; 100 mM Li^+ decreases the area per molecule by approximately 1 $Å^2$ at 30 mN/m. On the other hand, 10 mM Ca^{2+} induces decreases in area per molecule of 5–6 $Å^2$ at 30 mN/m (Demel et al., 1987). These results of the monolayer study are consistent with the present infrared data.

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Further Consideration of Flavin Coenzyme Biochemistry Afforded by Geometry-Optimized Molecular Orbital Calculations

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ABSTRACT: Investigation of lumiflavin and several other isoalloxazine ring derivatives has been carried out by geometry-optimized molecular orbital calculations. The results have provided insight into the flexibility of the flavin cofactor in the reduced and oxidized states, the regions of the fused three-ring system that should play an important role in flavin electron transfers, and the structural and functional role of the xylene and heteroatomic portions of the flavin system. The significance of these results is reviewed in relation to the experimentally identified chemical and biochemical properties of the flavin nucleotide coenzymes.

Flavin nucleotides are important biological cofactors that perform both single- and double-electron transfers. The isoalloxazine ring's accessibility to three oxidation states allows flavin coenzymes to couple the chemistry of two electron redox cofactors or organic molecules with molecular oxygen or cofactors transferring single electrons. The mechanism of dihydroflavin reaction with molecular oxygen has been studied extensively [for a review see Bruice (1984a,b)]. The protein environments of most flavin oxidases or monooxygenases enhance this reaction rate, yielding efficient oxidative catalysts or, in a special case, bioluminescence [for a review see Massey et al. (1980)]. In a variety of other proteins, interactions between the cofactor and its environment suppress the reoxidation of dihydroflavin by oxygen, which permits these flavoproteins to function effectively in electron transfers between proteins or between organic and inorganic substrates (Fox et al., 1982) and/or proteins. In addition to modifying chemical reactivity, interactions in the flavin nucleotide binding site can also profoundly affect the coenzyme's redox potential (Mayhew et al., 1975) or its accessibility to specific redox states (Ludwig et al., 1985).

Detailed information about the interactions of flavins with proteins and solvent can provide insight into the apoprotein's role in altering the geometric and electronic structures of the

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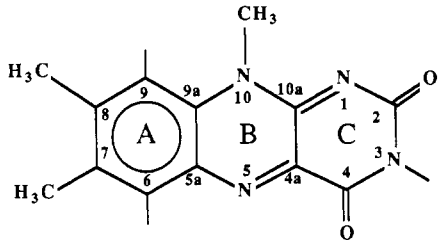

FIGURE 1: Molecular structure of oxidized lumiflavin (7,8,10-trimethylisoalloxazine) and the standard numbering for the ring atoms.

coenzyme as well as directing its biological function. Several studies have addressed these issues by different methods: X-ray diffraction (Schulz et al., 1982; Wierenga et al., 1983); NMR (Moonen & Müller, 1984; Moonen et al., 1984; van Schagen et al., 1981); reactivity of C(8) α-modified flavins (Krauth-Siegel et al., 1985; Zanetti et al., 1983). Amino acids in close proximity to flavin cofactors have been identified by reaction of proteins with amino acid selective reagents (Durfor et al., 1981), suicide substrates (Walsh, 1982), and derivatized flavins (Moore et al., 1978), although many times the role of these residues in modulating coenzyme reactivity is still unresolved. While these studies have enhanced the understanding of flavoprotein structure and function, the knowledge is far from complete concerning the effects on flavin redox chemistry arising from (1) flavin geometric constraints in proteins, (2) changes in charge distribution during flavin reduction, (3) the role of the flavin xylene ring, and (4) the role of flavin heteroatoms.

Several theoretical studies have sought to resolve further the electronic and geometric control of flavin coenzymes in nature (Dixon et al., 1979; Palmer et al., 1980; Teitell et al., 1980, 1981, 1982). The recent molecular orbital study by Hall et al. (1987) is the first thorough investigation of isoalloxazines using molecular orbital optimized geometries. All previous studies of the isoalloxazine system used structures derived directly from solid state or structures constructed from standard geometries or from related molecules. In the geometry-optimization approach an initial geometry is selected from the best available information, and the energy is computed for the initial geometry by use of molecular orbital methods; the geometry is then varied until the computed total energy reaches a minimum. Computed properties based on optimized geometry tend to be in better agreement with experimental values (Dewar, 1975). From the close agreement between physicochemical characterizations of flavins and the calculated molecular properties reported by Hall et al. (1987), we show that further insights into flavin structure and chemistry can be developed for the diverse role of flavins in flavoproteins.

Materials and Methods

The flavin molecular system was investigated through a molecular orbital study of methyl-substituted isoalloxazines (Hall et al., 1986). The primary focus of the investigation was lumiflavin (7,8,10-trimethylisoalloxazine, see Figure 1) in both its oxidized and two-electron-reduced (dihydro) forms. Several other neutral polymethylisoalloxazines were examined as well as protonated and deprotonated ionic species. Lumiflavin and related methylisoalloxazines were selected as models of flavin prosthetic groups because these simple isoalloxazines display the primary redox properties of the biological cofactors FMN and FAD (Walsh, 1978) and because considerable experimental data are available for comparison (Wang et al., 1973; Fritchie et al., 1975; Norrestam et al., 1972; Grande et al., 1977a,b; Eweg et al., 1980, 1982; Moonen & Müller, 1984; Moonen et al., 1984).

The MINDO/3 molecular orbital method with full geometry optimization was used in this study (Bingham et al., 1975). This is the first application of full geometry optimization to the flavin system. In the MINDO/3 program in the CHEMLAB system convergence of the optimization procedure is controlled by two factors: the change in the total energy and the change in atomic coordinates (gradient of the surface). For typical molecules in this study, the combination of tolerances on these quantities resulted in convergence on the heat of formation to within 1–5 kcal/mol. The inclusion of all atoms in the optimization means rather long execution times. A typical molecule in this study required about 24 h of CPU time on a VAX 11/780. In this study, bond distances in computed molecular structures generally agreed well with data from X-ray crystal structures, differing by 0.02 Å or less. Ionization potentials computed from MO[1] energy levels differed by 0.02–0.3 eV from those determined by UV photoelectron spectroscopy. These studies were confined to closed shell, ground-state molecules in the isolated gas phase. Numerical values for energies are given in kilocalories per mole for ΔH or in electronvolts for total energies and ionization potentials. The papers of Hall et al. (1987) include tabulated computed results for bond distances, conformation energies with respect to molecule folding, ionization potential, heat of formation, total energy, dipole moment, proton affinity, atomic partial charges, and enthalpy of reduction. For details of the methods, the reader is referred to Hall et al. (1987).

Results and Discussion

Molecular Structure and Conformation of the Flavin Isoalloxazine Ring System. An important aspect of the fused three-ring isoalloxazine molecular system is its flexibility about the N(5)–N(10) axis. Flexibility about this axis in reduced flavins has been invoked in the discussion of several aspects of flavin chemistry (Coles et al., 1977; Tauscher et al., 1973; van Schagen et al., 1980; Eweg et al., 1982). The π electron system of oxidized flavins formally contains 16 electrons: 1 from 12 of the 14 ring atoms and 2 each from the two pyrrole-like nitrogens N(3) and N(10). This 16-electron π system is not aromatic, although it has sometimes been incorrectly designated as such (Fritchie et al., 1975; Wang et al., 1973; Taushcer et al., 1973; Moonen et al., 1984). Interestingly, in discussions of protein-bound flavins, when attention has been directed to folding of the dihydro reduced form, there appears to be a tacit assumption that the oxidized form is much less flexible. Although Dixon et al. (1979) performed some (nonoptimized) calculations on folded geometries of oxidized lumiflavin, the complete conformational aspects of the oxidized form were not thoroughly investigated until the work of Hall et al. (1987).

Figure 2 presents the energy curves of conformation with respect to fold angle ϕ for both oxidized and two-electron reduced lumiflavin. These MINDO/3 computed energies indicate that both the oxidized and reduced forms are quite flexible. The most stable conformation of the oxidized form is computed to be planar (ϕ = 180°), whereas the lowest energy conformation of the reduced form is folded 27° from planarity (ϕ = 153°).

As previously suggested (Moonen et al., 1984; Dixon et al., 1979), the dihydro form is flexible; our computed results give a quantitative measure of the barrier to planarity of approximately 2 kcal/mol. Although the stable geometry is folded

[1] Abbreviations: MO, molecular orbital; HOMO, highest occupied molecular orbital; LUMO, lowest occupied molecular orbital; Fl, flavin in the oxidized form; FlH_2, flavin in the dihydro reduced form; EMF, electromotive force; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

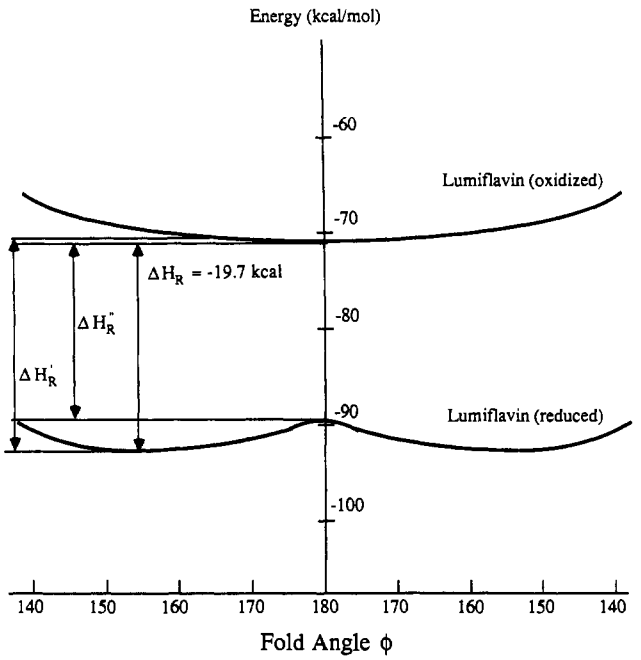

FIGURE 2: Conformation energy curves for lumiflavin. Energy (kilocalories per mole) computed as a function of fold angle, ϕ, for folding along the N(5)–N(10) axis, based on a small number of points (Hall et al., 1987).

as indicated by a minimum in the total energy, the computed electronic energy for the reduced form is at a minimum for the planar geometry. The folded geometry arises from decreased repulsion forces, especially in the regions around N(10)—CH_3···H—N(1) and N(5)—H···O=C(4). The reduced form is not antiaromatic as has been sometimes stated (Tauscher et al., 1973); the three-ring system contains 18 π electrons, which is more consistent with the $4n + 2$ Hückel aromaticity rule rather than any criterion for antiaromaticity. Drawing attention to the eight π electrons of pyrazine-like ring B and suggesting that its possible local antiaromaticity controls the conformation of the fused three-ring system (Tauscher et al., 1973) narrow the focus of attention too severely. The whole conjugated three-ring system should be taken into account.

On the other hand, the oxidized form contains 16 π electrons, which is not consistent with the $4n + 2$ Hückel rule. The calculated flexibility of the oxidized flavin indicates that only 1 kcal/mol is required to fold the molecule 10°, a flexibility consistent with the nonaromatic nature of the isoalloxazine system. This tendency toward antiaromaticity in the oxidized form may be the source of the low folding energy. The tendency toward nonplanarity in antiaromatic systems is, in this case, partially relieved by the delocalization of electron density into the carbonyl groups at C(2) and C(4).

Implications of these conformation computations are found in both the binding of flavin to proteins and the EMF of the flavin two-electron reduction. The protein environment of bound flavins interacts through hydrogen bonding and other somewhat less energetic nonbonding forces (Wiernga et al., 1979, 1983; Thieme et al., 1981) that are of sufficient strength to distort the isoalloxazine nucleus from its stable conformation in *both* the oxidized and reduced forms. As computed by Hall et al. (1987), ΔH for the two-electron reduction of lumiflavin is −19.7 kcal/mol (−0.854 eV). The energy change for this process is labeled ΔH_R in Figure 2. However, the protein environment can modulate this value by altering the fold angle ϕ of oxidized or reduced forms. If the oxidized form is folded a small amount, say 10°, then $\Delta H_{reduction}$ can be made more

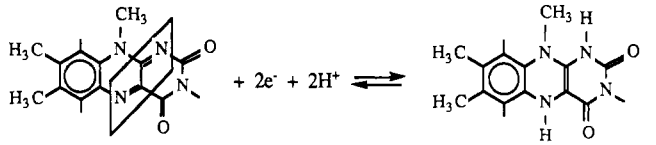

FIGURE 3: Two-electron reduction of lumiflavin emphasizing the diazadiene region in the box.

negative ($\Delta H_R'$) by about 1 kcal/mol (43 mV). In contrast, a less negative ΔH can be realized ($\Delta H_R''$) as the result of flattening the reduced form to planar geometry, changing ΔH by approximately 1.9 kcal/mol (83 mV). Thus, according to the MINDO/3 calculations, $\Delta H_{reduction}$ could vary approximately ±3 kcal/mol (±130 mV). The magnitude of this calculated modulation in EMF is similar to the experimental variation in flavoprotein reduction potentials (Moonen et al., 1984; Mayhew et al., 1975).

It should be noted here that two-electron reduction is computed to be spontaneous from the energy point of view: $\Delta H_{reduction} < 0$. While the reoxidation of FlH_2 with O_2 is spontaneous, the total energy of dihydroflavin is computed to be lower than that of the oxidized form. In order to obtain the total energetics (the free energy, ΔG), the entropy, ΔS, must also be known. For the two-electron (and two-proton) reduction reaction, it is expected that $\Delta S < 0$ because of the decrease in the number of particles upon two-electron reduction, as shown in Figure 3. Further, the reduced form may actually be a lower entropy state because it may be somewhat less flexible than the oxidized form, as indicated by the conformation energy curves in Figure 2. Also, changes in the solvation and hydrogen binding between the oxidized and reduced form can also alter ΔS. Unfortunately, it is not possible at this time to calculate accurately the entropy change upon reduction and, hence, it is difficult to compare directly formal potentials determined experimentally with the calculated ΔH values. However, because the electrode and solution effects are expected to be similar in related molecules, useful comparison can be made with computed energies and electrochemical potentials.

The nonaromatic nature of the oxidized flavin also presents the possibility that portions of the three-ring system may not be coplanar. Geometry optimization calculations by Hall et al. (1987) revealed that N(10) and its bonded methyl group in lumiflavin (and related methylisoalloxazines) are slightly out of the plane of the three rings. The other pyrrole-like nitrogen, N(3), and its attached hydrogen atom are, in contrast, found to be coplanar with the three-ring system. Further, the computed valence of N(10), 3.47, is greater than that for N(3), 3.28, indicating a greater degree of sp^3-like hybrid bonding for N(10). [The expected typical valence values are 4.0 for sp^3 and 3.0 for sp^2 carbon, nitrogen, and oxygen (Armstrong et al., 1973).] By comparison, the corresponding values for the pyridine-like nitrogens N(1) (2.99) and N(5) (3.09) clearly indicate sp^2 hybridization. These findings for N(10) suggest an electronic basis for the ^{15}N NMR results of Moonen et al. (1984), who found that N(10) behaves differently from N(3) in aprotic and protic solvents. Further characterizations of the nitrogen atoms will be discussed in a subsequent section.

Trimethyl substitution on ring A, especially including C(9), was found to introduce nonplanarity into ring A, based on the MINDO/3 optimized geometries. These results are fully consistent with the ^1H NMR data of Grande et al. (1977) and give some quantitative insight into the magnitude of the nonplanarity distortion suggested by the work of Grande. Such nonplanar distortions further indicate the lack of aromaticity

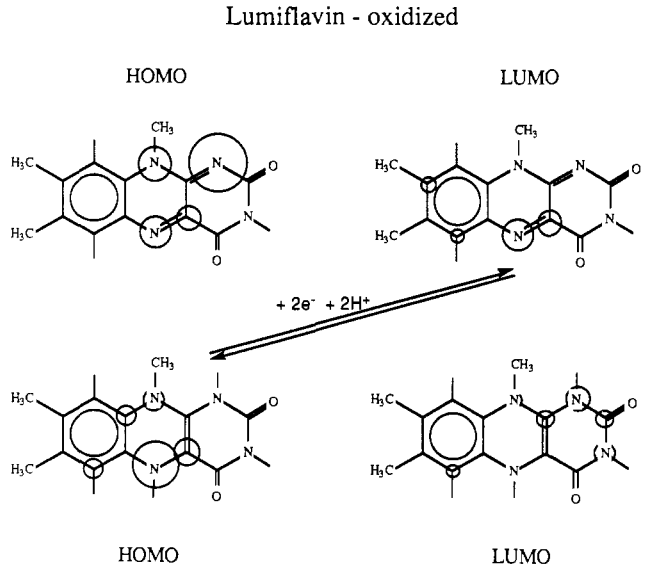

FIGURE 4: Representation of the molecular orbital coefficients for HOMO and LUMO of oxidized and reduced lumiflavin. The size of the circle indicates the relative importance of the orbital(s) centered on each atom. The double arrows indicate the relation between oxidized and two-electron-reduced lumiflavin.

of the isoalloxazine system and should be kept in mind when crystal structure data of flavoproteins are analyzed, especially when there are close contacts located in the vicinity of ring A.

Frontier Orbitals and Lumiflavin Redox Chemistry. The molecular structure basis of flavin redox chemistry is revealed by an examination of the frontier orbitals for the appropriate molecular species: the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO). The power of the frontier orbital concept lies in its combination of electron density (large molecular orbital coefficient) with energy related to reaction (MO energy of least magnitude). The frontier orbitals, then, described regions that are most accessible for electron donating or accepting. Two computed properties of interest in redox chemistry are the ionization potential, I_P, and electron affinity, E_A, which are computed as $I_P = -E(HOMO)$ and as $E_A = -(LUMO)$ according to Koopman's theorem (Koopman, 1934). Ionization potentials are more reliable when computed in this manner than are the electron affinity values (de Bruijn, 1978).

For oxidized lumiflavin the HOMO consists of a pair of nearly degenerate orbitals: a π MO located primarily in the diazadiene system and a nonbonding MO also located in the buta-1,3-diazadiene system, which emphasizes the lone pair on N(1). Interestingly, the π MO also includes some portions of ring A. The HOMO frontier orbital is shown in Figure 4. This finding and description of the HOMO is in contrast to CNDO/S results of Eweg et al. (1980), whose calculations found the HOMO to be a benzenoid type MO located primarily on ring A. Our results, however, agree with experimentally defined flavin redox chemistry, which involves the diazadiene region rather than ring A, as well as with the MO calculations of Teitell et al. (1980).

Computed ionization potentials agree closely with values from UV photoelectron spectra (Hall et al., 1987). The I_P values for oxidized and reduced lumiflavin suggest a stronger resemblance to phenazine and pyrrole than to lumazine and pyridine, which is also consistent with the suggested lack of conjugation of the O=C—NH—C=O moiety to the rest of the molecule (see below).

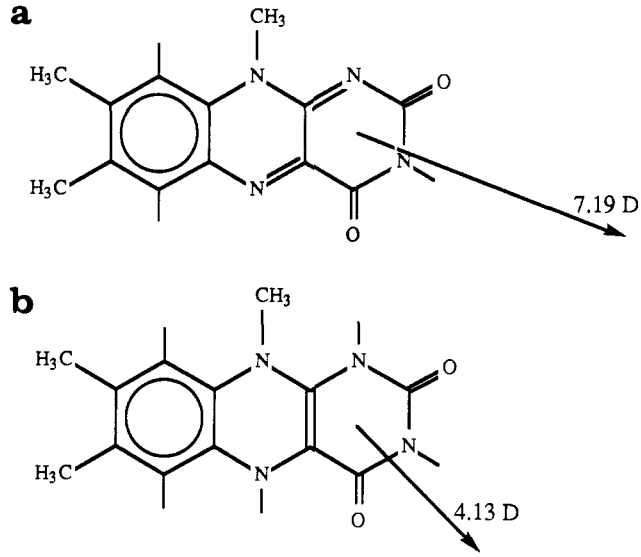

FIGURE 5: Computed dipole moment vectors for (a) oxidized lumiflavin and (b) dihydrolumiflavin.

The locus of electron accepting is determined by the nature of the LUMO, which for oxidized lumiflavin is a π^* MO also heavily concentrated in the diazadiene region, primarily on C(4a) and N(5) (Figure 4). Significant involvement of ring A is observed, especially on C(6) and C(8). The sign of the LUMO energy is negative, indicating a release of energy upon the accepting of an electron. This MO picture supports the belief that introduction of electrons into the isoalloxazine ring occurs at the N(5) region of the flavin.

Two-electron reduction of lumiflavin substantially alters the calculated flavin orbital structure. The addition of two electrons (and two protons on N(1) and N(5)) creates a new high-lying MO level separated by nearly 2 eV from the next highest occupied MO. The HOMO of reduced lumiflavin, which is the frontier orbital of interest in reoxidation, is a π MO located primarily on C(4a) and N(5) with some additional involvement on N(10) and ring A (C(5a), C(6), C(8), C(9a)). Based on the nature of the HOMO, the preferred site for electrophilic attack by a substrate should be C(4a) or N(5), which is consistent with the studies of Bruice (1984a,b) on the mechanism of oxygen reduction and with the inability of 5-deazaflavin to reduce oxygen. Thus the frontier orbitals of lumiflavin clearly indicate the importance of the diazadiene region in the redox chemistry of flavins. Both the oxidation and reduction of flavins are strongly associated with the N(1)=C(10a)—C(4a)=N(5) moiety. Additional insight into the role of ring A can be gained by studying the changes in electron density that occur upon flavin reduction.

Electron Distribution in Flavins. Analysis of the electron distribution in lumiflavin shows that ring C is the most polar portion of the molecule. The greatest polarity is associated with the two carbonyl groups. Since partial charges on atoms in rings B and C are relatively small, the dipole moment (Figure 5) is largely determined by the O=C—NH—C=O moiety in addition to N(1) and C(10a). The computed partial charges in both oxidized and reduced forms are in substantial agreement with the ^1H NMR work of Grande et al. (1977a). For details of electrons distributions see Hall et al. (1987).

In ring C the N(1) and N(3) atoms are the most negative ring atoms, while C(10a), C(2), and C(4) are the most positive. Upon reduction C(4a) becomes more negative and N(1) less negative, so that they are approximately of the same charge in the dihydro form. In contrast to these changes, the partial charges in the O=C—NH—C=O portion remain virtually

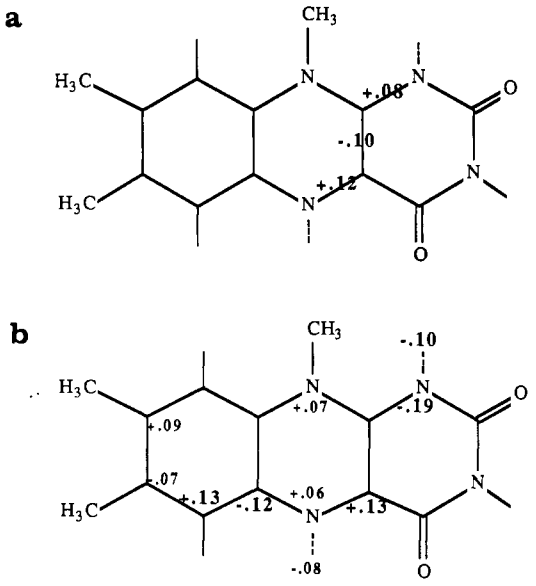

FIGURE 6: Changes in lumiflavin upon two-electron reduction: (a) Changes (greater than 0.04 Å) in the bond distances. + indicates increase in bond length; – indicates decrease in bond distance. (b) Changes (greater than 0.05 e) in the computed partial atomic charges. + indicates gain of electron density; – indicates loss of electron density. (Dashed lines to N(1)–H and to N(5)–H indicate that these atoms are only present in the reduced form.)

unchanged upon two-electron reduction, suggesting its electronic isolation from the rest of the conjugated system. Partial charge changes brought about by two-electron reduction are summarized in Figure 6. Whereas the geometry changes (Figure 6a) upon reduction are quite localized to the diazadiene region, changes in partial charges (Figure 6b) extend even into ring A, especially C(5a), C(6), and C(8) as well as N(10).

These computed changes in partial charges indicate a possible role for ring A in reduction/reoxidation even though it is apparently not directly involved in the redox biochemistry of flavins. The xylene-like ring A acts as a polarizability source for the changes occurring in the redox-active diazadiene region. This polarizability may simply facilitate the reduction and/or reoxidation of the flavin moiety, or the role of ring A may involve the fine-tuning modulation of the reduction potential brought about by the protein environment. For instance, in glutathione reductase there is an arginine residue located close to the C(8) methyl group (Thieme et al., 1981). The induced polarization caused by the positively charged arginine side chain could be involved in polarization through ring A and attendant changes at the redox center. This suggests that ring A and its methyl groups may play yet a different role in covalently bound flavins and also in those flavoproteins in which the methyl groups are more solvent exposed. These results indicate, then, that protein influence on ring A through effects such as polarization may be responsible in part for the versatility of flavins and that careful consideration should be given to the environment of ring A in the investigation of coenzyme binding sites of flavoproteins. Williamson has also called attention to this possibility (Williamson et al., 1985).

In contrast, similar MO calculations of 5-deazalumiflavin reveal quite a different picture (Hall et al., 1987). A major difference between lumiflavin and 5-deazalumiflavin is found in the changes that occur upon two-electron reduction. Changes in *both* bond distance *and* charge are localized in the diazadiene region in 5-deazaflavin. Thus, there is no involvement of ring A. The C(5) position becomes much more negative than does N(5) in the reduced flavin, and the change in partial charge at the 5-position is also much larger in the deazalumiflavin than in lumiflavin. Differences in structure and function between 1- and 5-deazalumiflavin are further discussed below in the light of the MO calculations.

Modification of Ring A Methyl Groups. The main route into C(8) α-modified flavin derivatives has been via the monobromo compound first isolated in good yield by Walker et al. (1972). Prior attempts at halogenation of flavins in acidic media led to substitution directly on the benzenoid ring at C(9) (McCormick, 1967). Establishment of the site of bromination was determined by ^{1}H NMR [assignments of the methyl resonances to C(7) α and C(8) α at δ 2.46 and 2.58, respectively (Walker et al., 1972) were done with the aid of model compounds with unambiguous assignments]. This shows that the bromination occurs only at the C(8) α-position with little or no C(7) α-brominated material formed. [It should also be noted that monobromination causes a large downfield shift of the remaining protons on the C(8) α to δ 4.72 as expected, but the C(7) α-methyl protons shift downfield only a small amount so that they coincidentally overlap with any remaining unbrominated C(8) α-methyl protons, at least on 90-MHz or smaller field spectrometers, giving the illusion that the upfield methyl group, C(7) α, has been brominated.] Under forcing conditions of excess bromine in pyridine, only the C(8) α-dibrominated species is formed, again with little or no C(7) α-bromination (McCormick, 1970).

This result is not expected since bromination of *o*-xylene yields the α,α'-dibromo compound with the $\alpha,\alpha,\alpha',\alpha'$-tetrabromo compound being formed with excess bromine. While the two methyl groups of flavin are in slightly different chemical environments (as shown by their ^{1}H NMR chemical shifts), this difference in electron density is not large enough to yield significant differences in the formation of the monobromo species versus the α,α'-dibromo compound. In fact, because the C(8) methyl group is more deshielded than the C(7) group, bromination should occur at C(7) α slightly easier than at C(8) α if the reaction occurs directly on the unmodified flavin by the expected free radical mechanism, which is indicated by the conditions.

Our calculations show the energy required for breaking a C–H bond from the C(7) methyl group to be the same as that for C(8). However, preliminary calculations also indicate that the radical centered at C(8) is more stable than the one at C(7) by approximately 0.5 kcal/mol (Hall et al., 1987). This effect is surprising, as it would be expected that each radical would be stabilized to a different extent due to resonance, but it may explain the monobromination.

Other complicating factors could also be implicated in this C(8) α-modification such as possible facile interconversion of the C(7) α to the C(8) α radical. Noncovalent dimers have long been known to occur with flavin solutions greater than 1–10 mM (Draper et al., 1968), and this might also lead to site-specific bromination. The possibility of an N(1)- or N(5)-bromo adduct [as is known for pyridine and bromine (Burti et al., 1966)] is another source of complications. Obviously, a complete study of the mechanism is necessary to answer these questions.

Evidence suggests C(8) α-modification of flavins to yield covalently attached flavoenzymes occurs through a methide tautomer centered at C(8) α as shown in Figure 7 (Hemmerich et al., 1959; Frost et al., 1980; Walsh, 1980). Facile C(8) α-tautomerization and the resultant interception by nucleophiles leads to C(8) α-modified flavins such as the histidyl-riboflavin complex (Walsh, 1980) or the morpholino derivative (Frost et al., 1980). Calculations of stability show that the

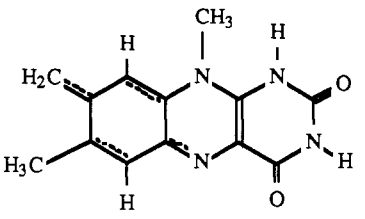

FIGURE 7: Quinone–methide tautomer of lumiflavin.

C(8) α tautomer is significantly more stable than the one centered at C(7) α (by 23 kcal/mol), clearly indicating that the C(7) α tautomer plays very little if any role in this chemistry.

Four Different Nitrogen Atoms. The fused three-ring isoalloxazine system contains four heteroatoms which, in the oxidized form, are usually classified as two pyrrole-like or α-type nitrogens, N(3) and N(10), and two pyridine-like or β-type nitrogens, N(1) and N(5) (Witanowski et al., 1972, 1981). In two-electron reduction the electrons and protons are formally added to the nitrogens of the N(1)–C(10a)–C(4a)–N(5) substructure, so that, in the reduced form, all four nitrogens might be considered pyrrole-like. However, ^{15}N NMR (Moonen et al., 1984) and MO calculations (Hall et al., 1987) show that all four nitrogens are indeed chemically different. Insight into these electronic properties as well as information about an atom's contribution to the HOMO and LUMO and to its polar environment can be gained from molecular orbital optimized geometry calculations. In this section these calculated properties and experimental information about each nitrogen are reviewed with an eye toward understanding the mechanisms of regulating the structure and diverse chemistry of flavin coenzymes.

The major importance of N(1) and N(5) is that they act as the center of the redox properties of the flavins, as indicated by the frontier orbitals. Changes in the local environment around these two nitrogens, as occurs in binding of the flavin to an enzyme, may result in relatively large changes in the redox potential of the flavin and could influence the stability of the semiquinone form (Massey et al., 1980). Other forces acting on the three-ring system, such as steric effects or hydrogen bonding, can also profoundly affect the flavin redox potential by hindering the folding of the molecule about the N(5)–N(10) axis.

The nitrogens of ring C, N(1) and N(3), are computed to be the most negative ring atoms in the isoalloxazine ring system. In contrast, the two ring B nitrogens are only slightly negative. It is clear, on the basis of partial atomic charge, that the four nitrogens behave rather differently. The two pyridine-like nitrogens, N(1) and N(5), change differently upon reduction: N(1) loses significant electron density but N(5) gains a little electron density. The two pyrrole-like nitrogens, N(3) and N(10), behave still differently. N(3) is barely changed upon reduction, whereas N(10) becomes somewhat more negative (see Figure 6). Thus, the partial charges on all four nitrogens are different and change differently upon reduction. These results indicate that the α/β nitrogen classification is not adequate for flavins. Further, these partial charge characterizations suggest that the hydrogen bonding of each nitrogen depends not only on the local environment within the enzyme but also on the partial charge of each nitrogen in each oxidation state.

N(1). The most basic nitrogen in oxidized flavin is N(1) in terms of both its partial charge and its prominence in the frontier HOMO, which is strongly distorted toward N(1). The N(1) nitrogen of the pyrimidine ring C exerts considerable influence in directing the reactivity of flavins. Proton removal from the reduced flavin's N(1) nitrogen accelerates the rate of oxygen and dihydroflavin reaction by 3 orders of magnitude (Eberlein et al., 1983). In flavoprotein oxidases, N(1) deprotonation by the protein environment is believed to enhance both the catalytic rate and reaction with sulfite as well as stabilize the anionic red flavin semiquinone (Massey et al., 1980). Molecular orbital geometry optimized calculations on oxidized flavin indicate that a large coefficient in the HOMO is found on the N(1) nitrogen. This implies that N(1) is the site of highest electron density in oxidized flavin and should therefore be the site of electron removal or proton addition. While oxidation of oxidized flavin is not often experimentally pursued, protonation of flavin in highly acidic media (pK_a = 0.18; Schollhammer et al., 1974) does occur. Crystal structure data clearly show that N(1) is the site of protonation (Tanaka et al., 1969; Trus et al., 1969).

Substantial changes in charge are calculated when N(1) of reduced flavin is deprotonated. Proton removal from N(1) significantly decreases the electron density at N(1), O(2), O(4), and C(4a) and to a lesser degree at C(6), C(8), and the C(8) α carbon, while the electron density increases at C(10a) and N(5). In natural systems this becomes important because deprotonation of N(1) in reduced flavin has a pK_a of 6.5. These calculated changes in electronic charge are interesting because N(1) deprotonation of reduced flavin results in a shift of electron density from N(1) to C(10a) and to a lesser degree from C(4a) to N(5). Thus, as N(1) of reduced flavin is deprotonated, the electron density begins to resemble the diazadiene bonding of oxidized flavin. This redistribution of electron density may aid in accelerating the reaction rate of anionic dihydroflavin with molecular oxygen. The calculated changes in charge density also affect the electron density in the xylene-like ring A. Thus, if changes at N(1) are propagated into ring A, then subtle changes in the polarization of ring A might conversely affect the deprotonation of N(1) or the reactivity of flavin with oxygen. Such effects may in fact explain the otherwise surprising presence of the arginine residue near the C(8) methyl in glutathione reductase (Thieme et al., 1981) and the iron–sulfur cluster proximity to ring A in trimethylamine dehydrogenase (Matthews et al., 1984).

Further insight into the role of N(1) in flavin chemistry can be gained by investigating the properties of 1-deazaflavin. Replacement of the N(1) atom with a carbon–hydrogen group makes the potential required for the two-electron reduction of 1-deazaflavin more negative by 75 mV (Spencer et al., 1977). While this illustrates the role of N(1) in determining the potential of flavin, both 1-deazaflavin and riboflavin can perform one- and two-electron transfers. According to the MO calculations, both oxidized and reduced 1-deazalumiflavin are less stable than lumiflavin, but the reduced form is destabilized more. Hence, the $\Delta H_{reduction}$ is less spontaneous (−15.15 kcal/mol). This effect may be attributed to the lack of conjugation to the 1-position in the reduced form. The HOMO of the reduced form is quite similar to that of lumiflavin, suggesting that redox properties of the two are similar. Also the extent of involvement of ring A is essentially the same.

Crystallographic studies on *p*-hydroxybenzoate hydroxylase (Wierenga et al., 1983) and glutathione reductase (Thieme et al., 1981) also suggest a structural role for the N(1) nitrogen and the C(2) carbonyl of isoalloxazine ring. In both proteins the N-terminus of long α-helices points at the N(1)/C(2) portion of the isoalloxazine ring. The large positive dipole associated with these α-helices not only stabilizes N(1) anions but also provides energy for noncovalent binding of the cofactor to the protein. Because N(1) is a hydrogen-bond acceptor in

the oxidized state but a hydrogen-bond donor in the reduced state, the binding of the coenzyme to the protein should be sensitive to flavin redox state and may also assist in directing flavoprotein reactivity (Hopner et al., 1976; Massey et al., 1984).

N(3). The N(3) proton is the most acidic in lumiflavin but plays no role in the frontier orbitals. While the partial charges on N(1) and N(3) of oxidized flavin are of similar magnitudes, the charge on the N(3) does not change upon flavin reduction. This observation and the lack of N(3) involvement in the flavin frontier orbitals suggest a limited role for N(3) in flavin redox chemistry. This prediction is consistent with the insensitivity of the flavin half-wave potential (Bruice, 1984a) and the optical absorption spectrum (Bootsma et al., 1984) to N(3) alkylation or acylation.

From our calculations, which show that the N(3)–H bond is the locus for the considerable dipole moment of the flavin ring system, it appears that the function of N(3) is primarily structural, as an anchor for flavin binding. This is also consistent with X-ray crystallographic analyses of flavin binding sites. Further, the reduction of flavin does not significantly alter the direction of the flavin dipole, but it does decrease its magnitude by 40%. This significant polarity change together with alterations in the hydrogen-bonding environment may be why some flavoproteins bind the reduced form of the coenzyme less tightly than the oxidized cofactor (Hopner et al., 1976). Previously, this change in flavin binding affinity was believed to be caused by alterations in the planarity of the flavin structure. Clearly, the present work suggests that the flexibility of both oxidized and reduced flavin plays a less restrictive role in coenzyme binding affinity.

N(5). As discussed above, C(4a) and N(5) are the primary contributors to the LUMO of oxidized flavin and should, therefore, be in the region of electrophilic attack. Interestingly, although the computed contributions of C(4a) and N(5) to the LUMO make them theoretically indistinguishable, the charge of N(5) is essentially neutral, whereas C(4a) is negative.

Experimentally, the direct transfer of a hydride unit to the C(5) of 5-deazaflavin bound to NADPH oxidoreductase, D-amino acid oxidase, and D-glucose oxidase as well as the formation of a ring adduct between the N(5) and C(4a) atoms after inactivation of several flavoenzymes by 2-hydroxy-3-butynoate (Walsh et al., 1972) is consistent with the present calculated properties of flavin. It is important to note, however, that the frontier orbital calculations indicate the region of electron accepting, which is only the first step in the electron-transfer chemistry of flavins and may not be the actual site for covalent bond formation that ultimately occurs.

C(4a) and N(5) display the two largest coefficients in the HOMO of reduced flavins, suggesting that this locus of the isoalloxazine ring system should play a large part in the nucleophilic attack of dihydroflavin. This result is consistent with the recent studies on the mechanism of dihydroflavin reaction with oxygen. Previous researchers also hypothesized a role for C(10a) and N(1) in the reoxidation reaction of flavins. The calculations analyzed herein are not consistent with that supposition, but the increased contribution of C(10a) and N(1) to the LUMO of reduced flavin does suggest that they could be important upon further reduction of flavin (e.g., addition of more than two electrons).

Comparison of the molecular properties of 5-deazaflavin, 1-deazaflavin, and lumiflavin illustrates that the former compound is not a "flavin-like molecule". Although the molecular HOMO and LUMO of oxidized flavin and 5-deazaflavin are similar, upon reduction the molecular HOMOs and LUMOs become significantly different. The most striking difference between reduced 5-deazaflavin and dihydrolumiflavin is the complete lack of involvement of the C(5) in the HOMO, which is most easily understood by the lack of a lone pair of electrons at the 5-position. Thus, while investigation of the molecular properties of 1-deazaflavin revealed insight into the role of N(1) in directing flavin chemistry, examination of 5-deazaflavin only illustrates that, without N(5), the facile transfer of one or two electrons by flavin is lost as is the rest of the characteristic isoalloxazine chemistry.

N(10). Structural connection between the isoalloxazine ring and the ribose side chain occurs at the N(10) nitrogen. NMR studies (Moonen et al., 1984) and the present MO study suggest that this nitrogen is slightly out of the plane of the isoalloxazine ring in the oxidized form. Why a nitrogen atom rather than a carbon atom at the 10-position? Clearly, the presence of a nitrogen at the 10-position allows conjugation of the entire ring system, but it also reduces the steric hindrance that would be introduced by a carbon–hydrogen bond in place of the nitrogen atom. The presence of a nitrogen at the 10-position permits three ring near coplanarity and also allows an increased polarizability of the isoalloxazine ring system. In addition, the presence of an N(10) nitrogen allows more flexibility to the flavin ring, leading to greater variability in flavin structure. This last feature results in greater diversity of coenzyme structure, which permits more flexibility in designing an enzymatic active site.

CONCLUSIONS

The molecular orbital optimized-geometry computations method was used in these investigations of flavins because of the expected improved quality of results. It is considered important to perform geometry optimization for the flavin system because the isoalloxazine system is a large, nonsymmetric, conjugated, fused three-ring moiety with nitrogens as heteroatoms. The three-ring system in oxidized form is not aromatic, but it is flexible and potentially nonplanar. The reduced forms are highly flexible and extensively conjugated. Small changes in the geometry in one part of the conjugated system can have an effect across the conjugated system, influencing planarity and molecular properties. These expectations are actually realized as is indicated by the high quality of the computed properties (Hall et al., 1987). These discussions are supported by the excellent agreement with experimental data as reported earlier (Hall et al., 1987).

From the MO calculations it is found that both oxidized and reduced states are highly flexible, requiring only 1–2 kcal/mol for significant deformation. The implications of these findings are important in both the modulation of flavin reduction potential and in protein binding. Although the four ring nitrogen atoms have generally been classified in two sets (pyridine-like and pyrrole-like), the calculations clearly show the diverse nature of all four nitrogen atoms. These characterizations shed light on the nature of their roles in the biochemistry of flavin molecules. Further calculations involving hydrogen bonding will investigate the detailed nature of its impact on the biochemistry of flavins.

Examination of highest occupied and lowest unoccupied MOs reveals the central role of the embedded diazadiene system in flavin redox biochemistry. Contrasts with deazaflavins highlight the role of various aspects of flavin structure. Finally, a new and hitherto little known role for the xylene-like ring A has been discussed on the basis of partial charge changes incurred upon two-electron reduction. The polarizability of ring A atoms may be important both in as-

sisting the redox of the diazadiene system as well as in the modulation of the redox EMF. In flavin reduction, molecular geometry changes are highly localized in the diazadiene system, but electronic changes extend to ring A. Contrasts with 5-deazaflavin suggest sources of its different behavior.

While it is clearly understood that many of the interesting aspects of flavin biochemistry take place in a hydrogen bonding solvent environment or by the complex constellation of effects at a protein binding site, these gas-phase molecule computations can be used with profit. Nonetheless, these limitations should be kept in mind. For these reasons in progress is a series of studies on hydrogen bonding and its impact on flavin properties and conformations.

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Enzymatic Hydrolysis of Short-Chain Lecithin/Long-Chain Phospholipid Unilamellar Vesicles: Sensitivity of Phospholipases to Matrix Phase State†

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ABSTRACT: Short-chain lecithin/long-chain phospholipid unilamellar vesicles (SLUVs), unlike pure long-chain lecithin vesicles, are excellent substrates for water-soluble phospholipases. Hemolysis assays show that >99.5% of the short-chain lecithin is partitioned in the bilayer. In these binary component vesicles, the short-chain species is the preferred substrate, while the long-chain phospholipid can be treated as an inhibitor (phospholipase C) or poor substrate (phospholipase A_2). For phospholipase C *Bacillus cereus*, apparent K_m and V_{max} values show that bilayer-solubilized diheptanoylphosphatidylcholine (diheptanoyl-PC) is nearly as good a substrate as pure micellar diheptanoyl-PC, although the extent of short-chain lecithin hydrolysis depends on the phase state of the long-chain lipid. For phospholipase A_2 *Naja naja naja*, both K_m and V_{max} values show a greater range: in a gel-state matrix, diheptanoyl-PC is hydrolyzed with micellelike kinetic parameters; in a liquid-crystalline matrix, the short-chain lecithin becomes comparable to the long-chain component. Both enzymes also show an anomalous increase in specific activity toward diheptanoyl-PC around the phase transition temperature of the long-chain phospholipid. Since the short-chain lecithin does not exhibit a phase transition, this must reflect fluctuations in head-group area or vertical motions of the short-chain lecithin caused by surrounding long-chain lecithin molecules. These results are discussed in terms of a specific model for SLUV hydrolysis and a general explanation for the "interfacial activation" observed with water-soluble phospholipases.

Phospholipases are small, water-soluble enzymes that catalyze the hydrolysis of phospholipid ester linkages. Phospholipase A_2 acts specifically on the *sn*-2 fatty acyl bond (Van Deenen & de Haas, 1964) while phospholipase C is specific for the phosphoglycerate bond of phospholipids (Little, 1981). These enzymes exhibit "interfacial activation" or a preference for substrate in aggregated forms (Pieterson et al., 1974; El-Sayed & Roberts, 1985). Furthermore, the type of phospholipid aggregate (e.g., bilayer, vesicle, or micelle) has a dramatic effect on the enzyme specific activity (Dennis, 1983; DeBose & Roberts, 1983). A number of hypotheses have been proposed to explain the observed kinetic trends. Several of these focus on aggregation-induced changes in the substrate (Brockerhoff, 1968; Wells, 1974, 1978; Apitz-Castro et al., 1979; Upreti & Jain, 1980). Other workers have attributed interfacial activation to phospholipid aggregation inducing conformational changes in the enzyme (Verger & de Haas, 1973; Roberts et al., 1977; Pluncknthun & Dennis, 1982). Yet another possibility is that the lipid matrix aggregation state may affect product release (El-Sayed & Roberts, 1985). Part of the problem in testing these different ideas is that different substrate chain lengths as well as physical aggregation states have been used.

To understand the different facets of interfacial activation of phospholipases, we have used a series of novel unilamellar vesicles made with short-chain lecithins and long-chain phospholipids as substrates for phospholipase A_2 (*Naja naja naja*) and phospholipase C (*Bacillus cereus*). The short-chain species in a micellar matrix are excellent substrates for these enzymes. How its hydrolysis rate will be affected when it is incorporated in a bilayer matrix of long-chain phospholipids, which is normally a poor substrate, should shed light on the reasons for the kinetic differences in micelles vs bilayers. Short-chain lecithin/long-chain phospholipid unilamellar vesicles (SLUVs)[1] have been characterized in considerable detail (Gabriel & Roberts, 1984, 1986, 1987). SLUVs are exceptionally stable and range in size from 150 to 1000 Å in diameter, depending on the phase state of the long-chain component.

Preliminary kinetic studies (Gabriel & Roberts, 1987) have shown that the short-chain lecithin is the preferred substrate for both phospholipase A_2 (~10-fold higher hydrolysis rates for the short-chain species over the long-chain component) and phospholipase C (~100-fold higher rates). In fact, hydrolysis of the short-chain lecithin proceeded at rates comparable to those for short-chain lecithin micelles, while the long-chain lipid in SLUVs was hydrolyzed at rates comparable to those

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[1] Abbreviations: SLUV, short-chain lecithin/long-chain phospholipid unilamellar vesicle; PC, phosphatidylcholine; diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; cmc, critical micelle concentration; T_m, midpoint temperature of the gel to liquid-crystalline transition of a long-chain phospholipid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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