Conformations and Electronic Structures of Oxidized and Reduced Isoalloxazine†

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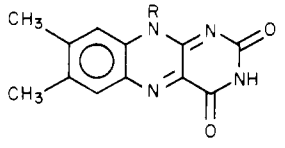
ABSTRACT: The conformations of oxidized and reduced isoalloxazine have been examined by a molecular orbital method, PRDDO (partial retention of diatomic differential overlap). The angle θ of fold about the N···N line of the central ring is zero for the planar oxidized form, but a bend of $\theta = 10°$ requires only 2 kcal/mol. On the other hand, the reduced form is nonplanar ($\theta \sim 15°$), and the barrier for reversal of this bend is 4 kcal/mol, comparable with that in simple amines. Molecular properties and reactivity are interpreted in terms of charge and orbital distributions, and localized molecular orbitals have been derived by the method of Boys.

The active cofactor for flavin enzymes, isoalloxazine, undergoes a wide variety of chemical transformations among the three oxidation states: oxidized, semireduced, and reduced (Bruice, 1976; Massey & Hemmerich, 1975; Bright & Porter, 1975). Besides electron and proton addition upon reduction, there is evidence for different intermediates under different conditions and for special electrophilic character at N_5 and C_{12} (i.e., C_{4a}: see Figure 1; Hemmerich & Schuman-Jörns, 1972). Other factors involved in the reactivity of this coenzyme are the widely different binding constants, in some enzymes, for the oxidized and reduced forms (Bright & Porter, 1975) and the question of constraints by the enzyme in forcing planarity on the reduced form of the flavin ring system (Ludwig et al., 1976) or nonplanarity on the oxidized form.

In this paper we address this last question in a theoretical calculation of the energetics of bending of the filled-orbital isoalloxazine molecules with **1** and **2** as models. In addition,



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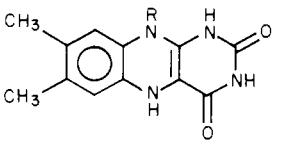

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we examine the charge distributions, indices of reactivity, and localized molecular orbitals in order to provide a theoretical basis for reactivity of these models and hopefully of the flavin cofactor in solution and in enzymes.

Experimental Procedure

Methods. Of methods recently reviewed (Schaeffer, 1976) applicable to complex molecules, we use here the approximation to a minimum basis set level self-consistent field method known by the acronym PRDDO [partial retention of diatomic differential overlap; Halgren & Lipscomb (1972, 1973)]. This method neglects or approximates a large fraction of the two-electron integrals but nevertheless yields energies and charge distributions in excellent agreement with far less efficient SCF methods in which all integrals are included (Halgren et al., 1978). These SCF methods are especially useful for the determination of molecular conformations (Allen, 1969), especially for small molecules (Radom & Pople, 1972; Stevens, 1974), and for rotational barriers (Pitzer & Lipscomb, 1963) because electron correlation corrections are small (Freed, 1968). Inversion barriers, which resemble the bend of isoalloxazine, are also given reasonably well, e.g., in the AH_3 type of molecule (Stevens, 1974; Dixon & Marynick, 1977; Marynick & Dixon, 1977). Our qualifications of these apparently successful results will be given below.

Less accurate theoretical methods have been directed toward spectral properties and partly to conformational problems in the isoalloxazines (Orf & Dolphin, 1974; Malrieu & Pullman, 1964; Sun & Song, 1973; Song, 1968). An extended Hückel calculation (Norrestam et al., 1969) of a substituted isoalloxazine showed a minimum energy at $\theta = 32°$ (Figure 1) and an abnormally large inversion barrier of over 3.8 eV (88 kcal/mol). A Pariser–Parr–Pople calculation on the reduced form **2** indicated that the molecule was folded about the N_5···N_{10} line (Fox et al., 1967).

Our calculations, by the PRDDO method, include all electrons in a minimum basis set of Slater orbitals having exponents of 1.2 for H1s, 5.7 for C1s, 1.625 for C2s and C2p, 6.7 for N1s, 1.95 for N2s and N2p, 7.7 for O1s, and 2.275 for O2s and O2p. The geometry of the reduced molecule was taken from the crystal structure of 9-bromo-1,3,7,8,10-pentamethyl-1,5-dihydroisoalloxazine (Norrestam & von Glehn, 1972) by replacing the methyl groups at N_1 and N_5 by hydrogens and the Br at C_9 by a hydrogen. The new hydrogens were placed along the appropriate N–R and C–R axes. All C–H distances were set at 1.10 Å and all N–H distances were set at 1.03 Å in order to correct for the effect of shortened bond distances which are observed in X-ray structures (Halgren et al., 1971). Tetrahedral bond angles were assumed for the hydrogen atoms of the methyl groups. The geometry of the oxidized form of isoalloxazine was obtained from the crystal structure of the lumiflavin–bis-2,3-diol trihydrate complex (Fritchie & Johnston, 1975). The C–H and N–H bond lengths were idealized as above, and methyl groups were given tetrahedral geometries. The hydrogen bonded to N_3 was taken in the molecular plane, and equal C_2–N_3–H and C_4–N_3–H bond angles were assumed. Calculations were initially performed on the crystal conformations for both the reduced form ($\theta = 30°$) and the oxidized form ($\theta = 0°$). The conformational analysis was performed by rotating the benzene ring fragment about the N_5···N_{10} axis in order to vary θ. Because the C_{11}–N_{10}–C_{17} and C_{14}–N_{10}–C_{17}

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‡ Received a Junior Fellowship from the Society of Fellows, Harvard University; A. P. Sloan Fellow (1977–1979).

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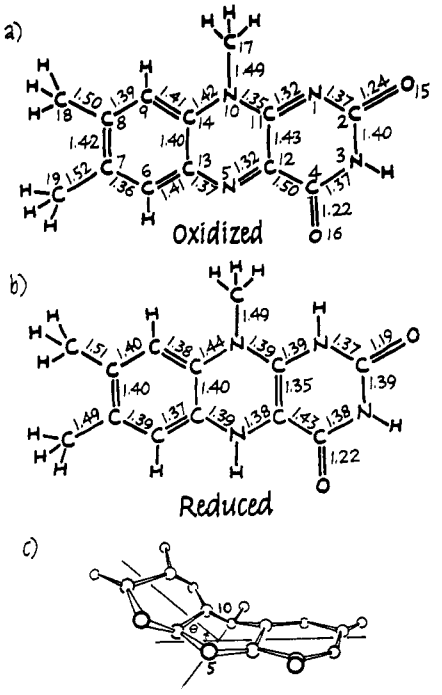

FIGURE 1: Valence bond description of the isoalloxazines showing bond lengths in angstroms and the numbering scheme. (a) Oxidized isoalloxazine; (b) reduced isoalloxazine; (c) isoalloxazine, heavy atoms only. The definition of θ is given for both molecules. θ is defined as the angle describing rotation of the plane containing the aromatic ring about the N_5–N_{10} axis with $\theta = 0$ being the planar molecule. Alternative numberings are C_{10a} for C_{11} and C_{4a} for C_{12}.

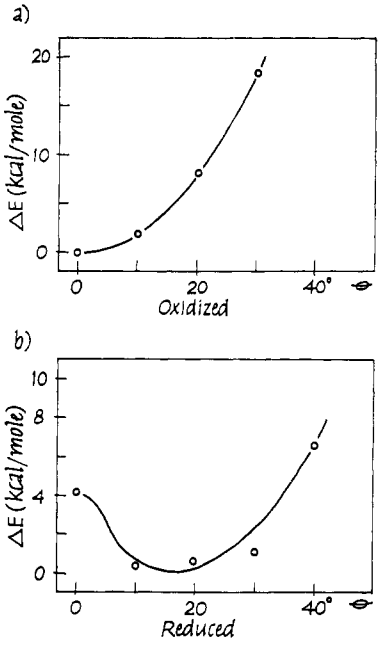

FIGURE 2: Conformational analysis showing the dependence of the energy on the bending angle, θ. Relative energies (kilocalories per mole) of oxidized isoalloxazine (a) are 0.0 (0°), 2.0 (10°), 8.5 (20°), and 18.6 (30°), while those for reduced isoalloxazine (b) are 3.7 (0°), 0.0 (10°), 0.2 (20°), 0.7 (30°), and 6.2 (40°). Reference energies are −542 760.9 kcal/mol for (a) at $\theta = 0°$ and −543 508.6 kcal/mol for (b) at $\theta = 10°$. The minimum energy for (b) is −0.5 kcal/mol at $\theta = 15°$, relative to 0.0 kcal/mol for $\theta = 10°$. Owing to the lack of geometry optimization in the remainder of the molecule, the points of Figure 2b lie somewhat away from the smoothed curve.

bond angles changed significantly upon variation of θ, they were reevaluated for each value of θ. The localized molecular orbitals were calculated by using the procedure of Boys (Kleier et al., 1974).

Table I: Molecular Properties

property	oxidized	reduced
ionization potential (eV)[a]	7.13	5.99
LUMO (eV)	2.29	4.87
dipole (D)	2.08	1.95
π manifold above σ orbitals (au)[b]	−0.262	−0.220
	−0.308	−0.315
	−0.347	−0.320
		−0.330

[a] As determined from Koopmans' theorem (1933) the ionization potential is the negative of the eigenvalue of the HOMO. [b] The most positive value is the HOMO. We report the eigenvalues at the π orbitals present before interleaving of the σ orbitals.

Table II: HOMO and LUMO Populations[a]

HOMO, ox		LUMO, ox		HOMO, red		LUMO, red	
N_1	0.54	N_5	0.45	N_1	0.14	C_4	0.10
C_7	0.12	C_6	0.19	N_5	0.51	C_7	0.23
N_{10}	0.13	C_8	0.26	C_8	0.13	C_8	0.20
C_{12}	0.13	N_{10}	0.12	N_{10}	0.24	C_{11}	0.46
C_{13}	0.17	C_{12}	0.32	C_{11}	0.20	C_{12}	0.21
O_{15}	0.36	C_{14}	0.13	C_{12}	0.29	C_{13}	0.24
		O_{16}	0.11	C_{14}	0.12	C_{14}	0.23
						O_{16}	0.16

[a] Populations ≥ 0.10 electron.

Results

Canonical Molecular Orbitals. Total energies (Figure 2) and conformational energy differences (Figure 2) show that the oxidized form should be planar, in qualitative agreement with the resonance argument. For an increase of θ from zero to 30°, the energy increases by about 19 kcal/mol, but, for a change from zero to 10°, the oxidized molecule is less stable by only 2 kcal/mol. Thus, a low-frequency bend of large amplitude is expected.

Our results for the reduced form differ from those of less accurate theoretical methods and from those of the oxidized form. The PRDDO method yields $\theta = 15°$, but only 1 kcal/mol is required as θ increases from 15 to 30°. The planar structure ($\theta = 0°$) lies only 4 kcal/mol above the equilibrium ($\theta = 15°$) conformation.

Molecular properties of the canonical (delocalized) molecular orbitals in Tables I–III include the ionization potentials, the populations in the highest occupied molecular orbital (HOMO), the virtual populations in the lowest unoccupied molecular orbital (LUMO) (Dixon et al., 1977), and the charge distributions and valences (Armstrong et al., 1973). Here (Table III), the group charge is the sum of the charge on C or N plus that of any bonded H atom. These group charges are more appropriate than atomic charges when comparison is made of C or N with and without attached H atoms; they also avoid overestimations of C–H or N–H polarities, in the sense of more positive H, as it occurs in Mulliken's population analysis (Mulliken, 1955). The bond overlap populations and degrees of bonding are shown in Figure 3.

Localized Molecular Orbitals. The LMO's are shown in Figure 4, where C–H and N–H bonds have been omitted. The bent bonds denote equivalent τ orbitals, which are favored relative to σ–π orbitals by the localization method of Boys. Thus, the aromatic ring has a Kekulé structure in both forms. Also, the reduced molecule has a set of τ bonds between C_{11} and C_{12}, as expected from simple valence theory (Table IV). A typical C–C τ bond is significantly bent away from the C–C axis, and its centroid of charge is displaced by 50° from the

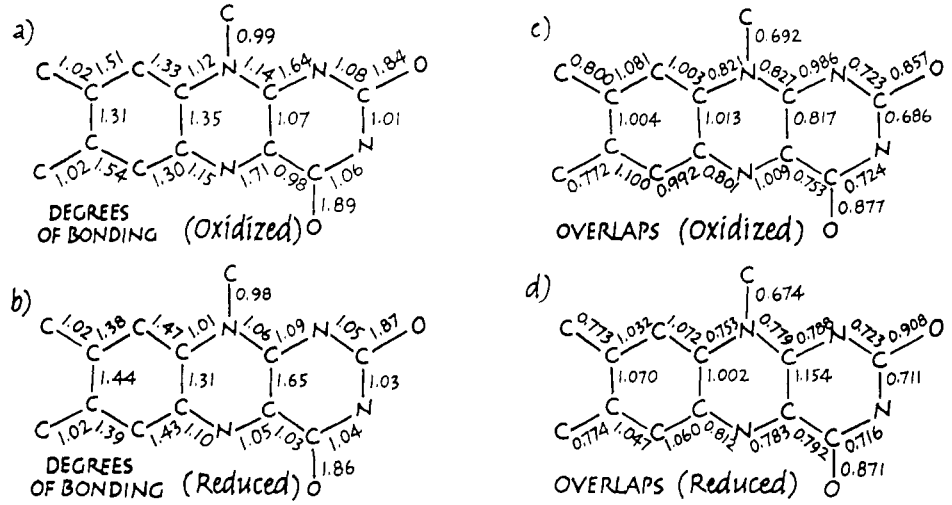

FIGURE 3: Degrees of bonding and overlap populations for the isoalloxazines. See Figure 1 caption for numbering. (a) Degrees of bonding, oxidized isoalloxazine; (b) degrees of bonding, reduced isoalloxazine; (c) overlap populations, oxidized isoalloxazine; (d) overlap populations, reduced isoalloxazine.

Table III: Charge Distributions and Valencies

atom	valency	anisotropy	charge	group charge
		(A) Oxidized		
N_1	3.10	0.50	−0.27	−0.27
C_2	3.97	0.02	0.24	0.24
N_3	3.37	0.28	−0.32	−0.10
C_4	3.97	0.02	0.22	0.22
N_5	3.07	0.62	−0.11	−0.11
C_6	3.99	0.01	−0.11	−0.04
C_7	4.00	0.00	0.01	0.01
C_8	3.99	0.00	0.06	0.06
C_9	3.99	0.01	−0.17	−0.04
N_{10}	3.54	0.21	−0.10	−0.10
C_{11}	3.99	0.01	0.18	0.18
C_{12}	3.98	0.02	0.02	0.02
C_{13}	3.97	0.02	0.02	0.02
C_{14}	3.99	0.01	0.13	0.13
O_{15}	2.17	0.61	−0.21	−0.21
O_{16}	2.19	0.61	−0.19	−0.19
C_{17}	3.95	0.04	−0.32	0.13
C_{18}	3.98	0.00	−0.41	−0.01
C_{19}	3.98	0.00	−0.41	−0.02
		(B) Reduced		
N_1	3.38	0.28	−0.29	−0.06
C_2	3.98	0.01	0.32	0.32
N_3	3.37	0.27	−0.31	−0.09
C_4	3.98	0.02	0.21	0.21
N_5	3.32	0.35	−0.27	−0.05
C_6	3.99	0.01	−0.18	−0.07
C_7	4.00	0.00	0.04	0.04
C_8	4.00	0.00	0.01	0.01
C_9	3.99	0.00	−0.14	−0.02
N_{10}	3.24	0.47	−0.16	−0.16
C_{11}	3.98	0.02	0.19	0.19
C_{12}	3.96	0.04	0.00	0.00
C_{13}	3.99	0.01	0.11	0.11
C_{14}	3.98	0.02	0.07	0.07
O_{15}	2.21	0.53	−0.27	−0.27
O_{16}	2.18	0.58	−0.23	−0.23
C_{17}	3.97	0.03	−0.32	0.06
C_{18}	3.98	0.00	−0.41	−0.03
C_{19}	3.98	0.00	−0.42	−0.03

internuclear axis. Also, hybridization for C–C τ bonds falls here between sp^3 and sp^5.

Polarization of the C_6–C_7 τ bond is opposite from that of the C_8–C_9 τ bond. The C_{11}–C_{12} τ bonds are also polarized opposite from that of the C_{13}–C_{14} τ bonds which are not quite equivalent (Table III). For comparison, anthracene has highly localized sets of τ bonds between C_6–C_7 and C_8–C_9, polarized

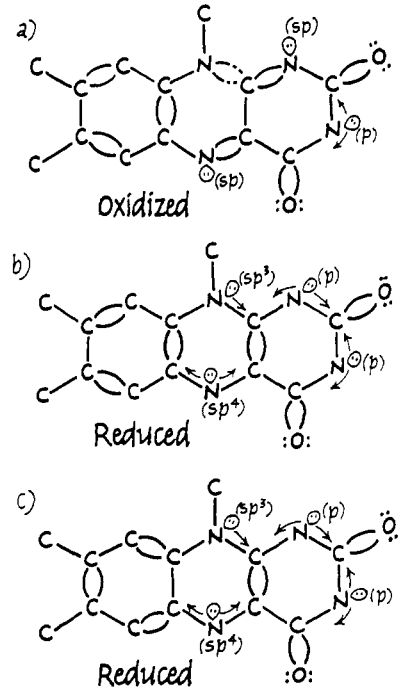

FIGURE 4: Localized molecular orbitals for the isoalloxazines. Bent bonds correspond to τ bonds above and below the molecular plane. Lone pairs on O are denoted by Lewis dots. Lone pairs on N are denoted by lobes with two dots. The hybridization of the lone pairs on N is shown together with the direction of delocalization of these lone pairs. (a) Oxidized isoalloxazine; (b) reduced isoalloxazine, one Kekulé structure for the aromatic ring; (c) reduced isoalloxazine, second Kekulé structure for the aromatic ring.

toward C_7 and C_8, respectively, and thus different from the τ bonds in reduced isoalloxazine.

Another difference from the fused aromatics lies in the existence on the LMO hypersurface of a second maximum corresponding to the other Kekulé structure for the ring. Multiple maxima have been found before (Dixon et al., 1977, 1978), but this result was unexpected. In anthracene, where the degree of bonding for C_6–C_7 is greater than that for C_7–C_8 or for C_6–C_{13}, the positions of large degrees of bonding agree well with the sets of τ bonds. On the other hand, in reduced isoalloxazine (Figure 3) we find the trends in degree of bonding as follows: C_7–C_8 > C_{13}–C_{14}; C_9–C_{14} > C_8–C_9; C_{13}–C_6 > C_6–C_7. Thus, the occurrence of two essentially equivalent LMO structures seems reasonable in retrospect, and significant

Table IV: Detailed Analysis of the τ Bonds

bond, A–B[a]	pop (A)[b]	pop (B)[b]	% s (A)[c]	% s (B)[c]	angle (A)[d]	angle (B)[d]
		Oxidized				
C_6–C_7	0.99	0.90	20	20	54	52
C_9–C_8	1.00	0.90	16	24	55	50
C_{13}–C_{14}	1.02	0.83	19	24	56	47
O_{15}–C_2[e]	1.10	0.90	10	20	51	59
N_5–C_{12}	0.98	0.98	21	17	49	59
N_1–C_{11}	1.13	0.84	15	21	56	53
N_{10}–C_{11}[f]	1.30	0.69	26	34	50	23
	1.30	0.37	11	23	73	43
		Reduced				
C_6–C_7	0.99	0.88	21	25	55	49
C_8–C_9	0.99	0.87	21	23	53	49
C_{12}–C_{11}	1.03	0.97	16	20	58	53
O_{15}–C_2[e]	1.15	0.85	9	21	53	58
C_{14}–C_{13}[f]	0.97	0.88	20	25	54	46
	1.02	0.79	18	22	58	58

[a] τ bond between atom A and atom B. [b] Population in bond due to A; population in bond due to B. Unless specified, both bonds are equivalent. [c] Percent 2s character in bond on A; percent 2s character in bond on B. [d] Angle of deviation (degrees) of centroid of charge (CC) from internuclear A–B axis: angle A = angle B–A–CC; angle B = angle A–B–CC. [e] The O_{16}–C_4 bonds are the same. [f] Nonequivalent τ bonds. Analysis for both bonds is presented.

aromatic character is indicated.

As expected, the τ bonds of CO in reduced isoalloxazine are slightly polarized toward O (Table IV), and the oxygen contribution is mostly p in character (sp^9). The lone pairs ($sp^{1.2}$) on O are well localized, but those on N vary. Lone pairs on N_1 and N_3 have 1.75 electrons from N, are mostly p (actually sp^9), and delocalize 0.10 electron to the adjacent relatively deficient C atoms. Hence, the C–N bonds are 10–12° away from the C–N axis. On N_5 the lone-pair LMO has 1.85 electrons from N, has therefore more s character (sp^4), and delocalizes 0.10 electron to C_{13} and 0.05 electron to C_{12} in agreement with the polarity of C_{11}–C_{12} and C_{12}–C_{13} (C_{13} has fewer electrons than C_{12}). On N_{10} the N population of 1.92 is an sp^3 hybrid which delocalizes only slightly toward C_{11}.

Oxidized isoalloxazine (Figure 4) has two fewer electrons and two less protons and thus a different bonding description. Polarization of the τ bonds in the aromatic ring makes C_6, C_9, and C_{13} electron rich and C_7, C_8, and C_{14} deficient. While the C_6–C_7 and C_8–C_9 bonds are oppositely polarized from those in anthracene, they are well localized so that no multiple maxima are found, and they have higher degrees of bonding than the remaining bonds of the aromatic ring. Probably, then, this ring is less aromatic in the oxidized form than it is in the reduced form of isoalloxazine.

In the oxidized molecule there are two sets of equivalent τ bonds and one set of unequivalent τ bonds between C and N. The N_5–C_{12} LMO's are nonpolar, while the N_1–C_{11} LMO's are polarized toward N. On the other hand, the bonds between N_{10}–C_{11} are unequivalent τ bonds due to delocalization of the lone pair on N toward the adjacent C. One of these bonds is substantially σ while the other is substantially π in character. This π-orbital LMO deviates by 72° from the bond axis, is mostly p (actually sp^8) on N, and has a substantial population of 1.52 electrons on N_{10}, suggesting that it originated from the lone-pair orbital. The lone pair on N_3 is pure p (sp^{99}), has a population of 1.75 electrons, and delocalizes by 0.10 electron to the adjacent carbons. The remaining lone pairs, on N_1 and N_5, are highly localized sp hybrids. Due to the presence of the C–N double bonds, these orbitals lie in the molecular plane and do not interact strongly with adjacent centers. In this respect, they are similar to the lone pairs on oxygen.

Discussion

Our conclusion, that the oxidized form is planar and the reduced form is nonplanar, is in agreement with the results of X-ray diffraction studies of similar molecules (Kierkegaard et al., 1971; Leijonmarck, 1977); we mention only the reduced forms for which the angles of bend θ are 32, 31, 36, 8.4, 30, 17, and 32° in the order of reference (Norrestram et al., 1969; Norrestram & von Glehn, 1972; Werner & Rönnquist, 1970; Werner et al., 1971; Leijonmarck & Werner, 1971; Norrestam, 1972; von Glehn et al., 1977). The 8.4° angle occurs in 5,5-diethyl-3,7,8,10-tetramethyl-1,5-dihydroisoalloxazine, where the diethyl substituents may favor sterically less distortion. The 17° binding angle in 4a-allyl-3,5,7,8,10-pentamethyl-4a,5-dihydroisoalloxazine is rumpled in shape because of the tetrahedral bonding at C_{12} (i.e., 4a). Thus, the unhindered reduced form probably prefers a bending angle of about 32°. However, in the reduced form of the propyl-linked flavin–nicotinamide bis coenzyme crystals (H_2Fl_{red}–C_3–Nic^+)NO_3^-·$4H_2O$ of formula $C_{21}H_{30}N_7O_{10}$, the bending angle is only 12.7°, probably due to the unique stacking forces in this crystalline form (Porter et al., 1977; Porter & Voet, 1978). Thus, deformation occurs readily with little energy cost in the reduced isoalloxazine ring.

Although the inversion barrier at $\theta = 0°$ is only 4 kcal/mol in our isolated reduced isoalloxazine, the NMR barrier is about 13 kcal/mol in aqueous solution and about 10 kcal/mol in acetone (Tauscher et al., 1973). One possibility for this discrepancy between our results and the NMR values is that substituents at C_6, and possibly at N_1 and N_{10}, may influence the NMR barrier. Another possibility is that our atomic basis sets are inadequate. If our curve (Figure 2b) is rescaled to a barrier of 13 kcal/mol, the energy required for a flattening from 15 to 5° is 8 kcal/mol. We shall return to this result below in connection with the X-ray results on flavodoxin. Examples of inversion barriers, in kilocalories per mole, are 5.8 for NH_3, 4.8 for H_2NCH_3, 4.4 for $HN(CH_3)_2$, and 6.0 for $N(CH_3)_3$ (Rauk et al., 1971). In NH_3, a minimum basis set gives a barrier which is high by only 20%. However, this calculated barrier is extremely sensitive to increases in the size of this basis set and tends to widely different values until polarization functions are included in a larger set (Stevens, 1974). At present, it is quite impractical to employ such a large basis set on a molecule as large as isoalloxazoline, so we have to be content with only a qualitative, and some semiquantitative, interpretation of our results.

In relating these calculations to flavodoxin (Burnett et al., 1974; Ludwig et al., 1976), we first note that further refinement of the reduced form leaves the isoalloxazine ring with an angle of bend θ of about 5° (M. L. Ludwig, personal communication). In our calculation (Figure 2b) this 15 to 5° change would cost 3 kcal/mol, and, if this curve is rescaled to the NMR results, the cost is 8 kcal/mol favoring the oxidation. However, there are other dominant effects. If we compare FMN(semiquinone) $\rightleftarrows$ FMN(reduced) for which E_0' is −0.172 V with flavodoxin(semiquinone) $\rightleftarrows$ flavodoxin(reduced) for which E_0' is −0.399 V, we see a large effect of the protein. (We did not consider the semiquinone in our calculations.) A dominant effect here is reflected in the effect of the protein interactions as shown by the binding constants of 7×10^{11} M^{-1} for FMN(semiquinone) and 1.0×10^8 M^{-1} for FMN(reduced) when binding to the apoprotein (Bright & Porter, 1975; M. L. Ludwig, personal communication, 1978). Thus, binding of the semiquinone is favored by 5.3 kcal/mol,

reflected in the change of E_0' above. Furthermore, the clear evidence that the planar FMN(oxidized) form is bound to the apoenzyme considerably less strongly ($K = 2.3 \times 10^9$ M^{-1}) than the semiquinone form suggests that there are important differences in the detailed interactions between the apoprotein and these three states of FMN. Finally, there is an effect of local conformational change of the protein structure, yet to be described in detail, when FMN becomes fully oxidized. It is generally recognized that these other effects need to be taken into account, in addition to considerations of planar constraint of the isoalloxazine ring, in flavodoxin (James et al., 1973).

In succinate dehydrogenase, the flavin is covalently bound to the enzyme, so that the potential is not modulated partly by dissociation constants. Here, the redox potential of flavin in the inactive (oxaloacetate) enzyme, −0.19 V, is comparable to that of free flavin in solution. In the active enzyme the redox potential is shifted to about zero (6 ± 19 mV) (Gutman, 1978). This change of 0.20 V, equivalent to 4.6 kcal/mol, has been attributed to distortion of the planar oxidized form of the isoalloxazine ring to the butterfly form of the reduced state (Gutman, 1978). Moreover, dramatic changes in the CD spectra were not observable following deactivation. Thus, the oxidized state is destabilized to a level which favors reduction by succinate. Again, the energetics are consistent with the effects noted above, but the detailed analysis may be much more complicated and may have to await X-ray diffraction studies of the interactions of the cofactor with the enzyme and of conformational changes of both enzyme and flavin upon oxidation and reduction.

On the other hand, in L-amino-acid oxidase the reversible inactivation is accompanied by very large changes in the CD spectrum of the oxidized isoalloxazine and by a shift in redox potential. This may be another example in which the protein can modulate the redox potential by influencing the geometry of the flavin ring system (Coles et al., 1977). Whether this effect is more general remains to be seen (Tauscher et al., 1973).

In the remainder of this discussion, we refer to the canonical (delocalized) molecular orbitals. While the use of static, ground state indices of electrophilic and nucleophilic attack has severe limitations, we attempt some predictions and correlations based primarily on π-orbital densities in the highest occupied and lowest unoccupied molecular orbitals, as in the aromatic hydrocarbons (Dixon et al., 1978). Here, we use group charges, exclude the C=O group, and suggest electrophilic (nucleophilic) attack at negatively (positively) charged atoms or at atoms which have the highest actual (virtual) population in the HOMO (LUMO).

For oxidized isoalloxazine, N_1 is predicted by both orbital and charge criteria to be the preferred site for electrophilic attack. Carbons C_{12} and C_{13} (orbital criterion) or C_9 and C_6 (charge criterion) are suggested to be the carbons at which electrophilic attack will occur. For nucleophilic attack, the orbital criterion gives N_5 first followed by C_{12}, while charges suggest C_{11} followed by C_{14}.

For the reduced molecule, we predict that electrophilic attack should occur at N_5, followed by C_{12}, using the orbital criterion while the charge criterion gives N_{10} and C_6 as the most likely sites. Both criteria give C_{11} as the predicted site for nucleophilic attack, followed by C_{13}. Using more complicated indices calculated from extended Hückel and Pariser–Parr–Pople wave functions, Sun & Song (1973) find that C_{11} is the predicted site for nucleophilic attack. One set of the indices employed by Sun and Song yielded N_5 and C_{12} as the most likely sites of electrophilic attack while another set gave C_{11} and C_{12} as the likely sites. Using extended Hückel theory, Orf & Dolphin (1974) predicted electrophilic attack at C_{12}, followed by C_{11}. Although we agree with Sun and Song on the site for nucleophilic attack, there is some discrepancy on the predicted site of electrophilic attack between the different methods. Thus, some care should be taken in using these indices, especially of semiempirical methods, to predict reactivity. On the experimental side, N_5 and C_{12} (i.e., C_{4a}) have been singled out as highly electrophilic sites (Hemmerich & Schuman-Jörns, 1972). However, no single mechanism covers all examples (Bruice, 1976; Walsh, 1978). Some adducts which have been identified involve general acid-catalyzed attack at C_{12} (Yokoe & Bruice, 1975; Loechler & Hollocher, 1975) or an N_5 adduct when nitroalkanes are oxidized by D-amino-acid oxidase (Porter et al., 1972, 1973). Also, the FMN of luciferase has been shown to form an adduct at C_{12} (i.e., C_{4a}) (Ghisla et al., 1978). These results are not inconsistent with the orbital criterion as a static index of reactivity.

Our estimate of the ionization potential of the oxidized form from Koopmans' theorem (Koopmans, 1933) is 7.13 eV. This value may be in error by about 1 eV, but it is much lower than values given by the extended Hückel (11.98 eV), CNDO (10.75 eV), and Pariser–Parr–Pople (9.7 eV) methods (Song, 1968). Also, the dipole moments of about 2 D (Table I) are probably too large because of the use of a minimum basis set, but we suspect that the CNDO prediction of 4.8 D is much too large (Song, 1968).

Finally, we comment on atomic valences, normally 4, 3, and 2 for C, N, and O, respectively (Armstrong et al., 1973), and on anisotropies, normally large when lone pairs dominate (0.43 for N in NH_3, 0.63 for O in H_2CO, but only 0.16 for C in H_2CO). In oxidized isoalloxazine normal valencies prevail, except for N_3 and N_{10} which have valencies greater than 3 and less anisotropy than N_1 (Table III). Thus, the lone pairs on N_3 and N_{10} participate in extra bonding. In reduced isoalloxazine, only the four nitrogen atoms differ from their simple valence; they have valences higher than 3, with decreased anisotropies consistent with lone-pair involvement in bonding.

Degrees of bonding are clearly superior to overlap populations for describing bonding (Figure 3), because they correlate more securely with bond multiplicity and are more nearly independent of atom type than are overlap populations (Armstrong et al., 1973). For values of degrees of bonding falling between 1 and 2 for C–C bonds, a benzenelike delocalization is expected; in benzene the degree of bonding is 1.44 for a bond length of 1.40 Å (Kleier et al., 1975). Degrees of bonding indicate less aromatic character in the aromatic ring in the oxidized form as compared with reduced isoalloxazine. For example, the C_6–C_7 and C_7–C_8 bonds of the oxidized form have more double-bond character, correlating with shorter lengths and increased degrees of bonding. The C_{11}–C_{12} bond in the reduced form has less double-bond character (1.65) than the normal double bond (2.00). Similarly, the N_5–C_{12} and N_1–C_{11} bonds of the oxidized molecule have diminished double-bond character.

Acknowledgments

We acknowledge helpful comments from M. L. Ludwig, D. Voet, and M. Gutman.

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