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Evidence for α-Keto-enol Tautomerism in Reduced Acetylisoalloxazines

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The spectra of fully reduced 6- and 8-acetyl-10-methylisoalloxazine display anomalously intense absorptions near 500 nm that are not characteristic of the expected 1,5-dihydro reduction products. The reduced absorbing species are characterized as α-enol tautomers on the basis of correspondence with predictions from INDO/S spectral calculations. The spectral calculations used geometries optimized within the framework of the semiempirical MNDO method.

INTRODUCTION

Flavin analogs **1–3** were synthesized in the course of studies, by Kaiser and coworkers, of flavopapains, semisynthetic enzymes formed by covalently attaching a flavin coenzyme analog to the active site of the protein papain (*1*). The enzymatic activities of such semisynthetic flavopapains can be quite high, probably as a result of hydrogen bonding between the acetyl carbonyl and the peptide backbone which favorably positions the flavin within the hydrophobic binding site of the protein (*1b,c*).

The present study was prompted by the observation that the absorption spectra of the two-electron reduction products of the flavin derivatives **1** and **3** have features, principally a strong visible absorption near 500 nm, that are not characteristic of the expected reduction product, 1,5-dihydroflavin (*1c*). A similar spectrum has also been reported for a related system, reduced 8-formyltetraacetylriboflavine (*2*). The unusual absorption properties of these reduced flavin derivatives led us to perform all-valence-electron semiempirical MO-CI calculations of the absorption spectra and electronic structures of **1**, **2**, and **3** and their possible reduction products with the goal of characterizing the reduced species responsible for the observed absorptions.

The experimental picture for oxidized and reduced flavins can be briefly summarized as follows. The strong absorptions near 450 and 370 nm characteristic of oxidized flavins are typically bleached by full (two reducing equivalents) reduction of the flavin nucleus. The resulting 1,5-dihydroflavins thus usually have much less absorption intensity in the region above 300 nm, with a typical spectrum

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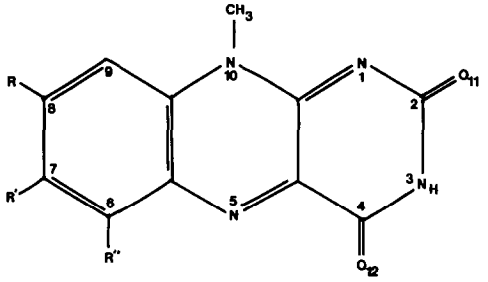
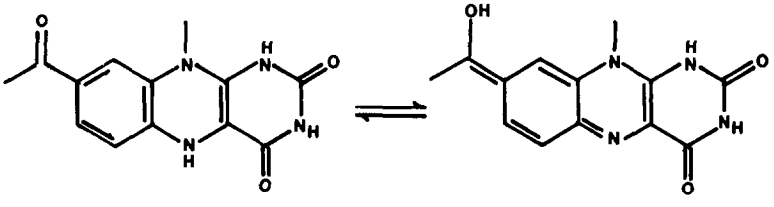


FIG. 1. Structures of oxidized *x*-acetyl-10-methylisoalloxazines: **1**, $x = 8$, R = CH_3CO, R′ = R″ = H; **2**, $x = 7$, R′ = CH_3CO, R = R″ = H; **3**, $x = 6$, R″ = CH_3CO, R = R′ = H.

consisting of broad, overlapping bands of decreasing intensity near 295, 350, and 400 nm (*3*). The particular shape of this spectrum shows much variability between reduced flavins, however, due to differing ring conformations among reduced flavin derivatives. The conformation of the flavin nucleus can change greatly upon full reduction, with the nearly planar geometry of an oxidized flavin becoming bent in a butterfly conformation about the N5-N10 axis in the reduced form (*4*). Experimental evidence for bent conformations of 1,5-dihydroisoalloxazine derivatives comes from both X-ray studies of crystal structures (*5*) and spectral studies of solutions (*6*). Folding angles of 30–35° are commonly observed, but not all fully reduced derivatives show such a large deviation from planarity. (For that matter, even some oxidized flavins are distorted out of plane by as much as 4° (*5*).) The spectra of bent derivatives tend to show more structure than those of the planar derivatives, with the 350-nm peak more prominent than the longer wavelength feature (*3*).

The possible products resulting from the addition of two reducing equivalents of hydrogen to **1–3** include both the usual 1,5-dihydro product (e.g., **4**) and also the α-enol tautomer (e.g., **5**), which is formed by migration of a proton from N5 to acetyl oxygen. Reduction of the isoalloxazine ring by addition of hydrogen at positions other than N1 and N5 is not expected to be significant under the conditions employed by Slama *et al.* (*1c, 3*).

Recent theoretical studies of flavins have tended to focus on the electronic structure of ground state species. Studies along these lines include *ab initio* MO studies of isoalloxazines (*7*), MINDO/3 MO calculations of oxidized (*8*), and reduced flavins (*9*), and of the active site of flavodoxin (*10*), and an Extended



4 **5**

FIG. 2. α-Keto–enol tautomerism.

Hückel study of flavin reactivity as a cofactor for monoxygenases (*11*). CNDO/S MO calculations have been used to assign the photoelectron spectra of alloxazines and isoalloxazines (*12a*). A PRDDO conformational analysis of oxidized and reduced isoalloxazine (*13*) found a minimum in the potential curve at 15° for bending in the reduced species that is rather shallow, consistent with the observation that small changes in structure or environment can have a large effect on the extent of nonplanarity.

More closely related to the present work are studies that have employed semiempirical CI methods to study electronically excited states of flavins. The PPP-CI method has been used to calculate the transition energies and strengths of excited states of lumiflavins (*14*) and to model spectral shifts induced in oxidized isoalloxazine by hydrogen bonding with protein residues (*15*). The present study appears to be the first all-valence-electron calculation of absorption spectra of flavin derivatives (*16*).

METHODS

Electronic properties were calculated with the all-valence-electron INDO/S method, which has been described in detail elsewhere (*17*). Two-center repulsion integrals were evaluated by an empirical Mataga–Nishimoto–Weiss formula (*17f, g*). Transition energies and intensities were obtained from CI wavefunctions constructed from a basis of 197 configurations, which included all single excitations between the 14 highest occupied MOs and the 14 lowest unoccupied MOs.

Molecular geometries for the INDO calculation were obtained by constructing a geometry for each species using standard bond lengths and angles (*18*) and then using this "standard" geometry as the starting point for a total geometry optimization using the semiempirical MNDO method (*19*). Total geometry optimization was considered preferable to using X-ray structures of related compounds as inputs to the spectral calculations, since, as mentioned above, the geometry of the reduced flavins is quite variable and sensitive to the presence of substituents. Although MNDO has been carefully parameterized using a large number of molecules with known structures to yield reliable geometries and relative energies for a variety of organic compounds, we will find in the discussion below that we are led to discount its predictions concerning the positions of the tautomeric equilibria in the flavin analogs **1–3**.

RESULTS AND DISCUSSION

MNDO optimized geometries. Calculated bond lengths are displayed in Table 1 for the three forms of the 6-, 7-, and 8-acetylisoalloxazines: oxidized, reduced α-keto, and reduced α-enol. Bond lengths calculated for the flavin nucleus in the oxidized and reduced α-keto form were consistently larger than those from X-ray data for flavin derivatives in the corresponding oxidation states, but generally agreed within 0.04 Å, while bond angles (Fig. 3) were typically within 2° of experi-

mental values (5). Slightly larger bond angle discrepancies of 3–5° were observed for the carbonyl carbons of the isoalloxazine ring.

The fused rings of the oxidized isoalloxazines are all calculated to lie in a plane (Table 2), in agreement with observation. The results for the reduced species are more varied, as is to be expected from the experimental and theoretical results

TABLE 1

MNDO OPTIMIZED BOND LENGTHS (IN ÅNGSTROMS) (INDO π-BOND ORDERS)

8-Acetyl-10-methylisoalloxazine			
		Reduced	
	Oxidized	α-Keto	α-Enol
N1—C10a	1.31 (0.74)	1.40 (0.36)	1.40 (0.36)
C10a—C4a	1.48 (0.33)	1.38 (0.81)	1.39 (0.76)
C4a—N5	1.30 (0.84)	1.43 (0.21)	1.40 (0.37)
N5—C5a	1.40 (0.41)	1.42 (0.20)	1.31 (0.77)
C5a—C6	1.42 (0.58)	1.41 (0.62)	1.47 (0.42)
C6—C7	1.39 (0.72)	1.40 (0.63)	1.36 (0.84)
C7—C8	1.42 (0.59)	1.41 (0.63)	1.48 (0.39)
C8—C9	1.41 (0.68)	1.41 (0.62)	1.48 (0.34)
C9—C9a	1.43 (0.61)	1.41 (0.63)	1.37 (0.82)
C9a—C5a	1.43 (0.59)	1.43 (0.59)	1.49 (0.39)
C9a—N10	1.42 (0.37)	1.45 (0.15)	1.44 (0.17)
N10—C10a	1.41 (0.47)	1.43 (0.20)	1.41 (0.33)
C8—C(acyl)	1.52 (0.28)	1.51 (0.09)	1.48 (0.79)
(C—O)acyl	1.23 (0.86)	1.22 (0.86)	1.36 (0.31)
7-Acetyl-10-methylisoalloxazine			
		Reduced	
	Oxidized	α-Keto	α-Enol
N1—C10a	1.31	1.40	1.41 (0.27)
C10a—C4a	1.48	1.38	1.45 (0.58)
C4a—N5	1.30	1.41	1.31 (0.69)
N5—C5a	1.40	1.40	1.40 (0.38)
C5a—C6	1.42	1.41	1.38 (0.77)
C6—C7	1.41	1.42	1.47 (0.38)
C7—C8	1.42	1.41	1.47 (0.41)
C8—C9	1.40	1.41	1.36 (0.82)
C9—C9a	1.42	1.41	1.45 (0.46)
C9a—C5a	1.43	1.44	1.47 (0.42)
C9a—N10	1.41	1.44	1.38 (0.60)
N10—C10a	1.41	1.42	1.38 (0.44)
C7—C(acyl)	1.51	1.51	1.39 (0.78)
(C—O)acyl	1.23	1.23	1.36 (0.28)

TABLE 1—*Continued*

6-Acetyl-10-methylisoalloxazine			
		Reduced	
	Oxidized	α-Keto	α-Enol
N1—C10a	1.31	1.40	1.40
C10a—C4a	1.48	1.38	1.39
C4a—N5	1.30	1.43	1.39
N5—C5a	1.40	1.44	1.31
C5a—C6	1.44	1.42	1.49
C6—C7	1.41	1.41	1.47
C7—C8	1.41	1.40	1.36
C8—C9	1.39	1.41	1.45
C9—C9a	1.43	1.41	1.37
C9a—C5a	1.44	1.43	1.50
C9a—N10	1.42	1.44	1.44
N10—C10a	1.40	1.42	1.40
C6—C(acyl)	1.52	1.51	1.39
(C—O)acyl	1.23	1.22	1.36

cited above, with some species predicted to be planar and others bending in the butterfly conformation. The dihedral angles of the reduced 6- and 8-acetyl keto tautomers are 25° and 30°, respectively, while the 7-acetyl keto tautomer is predicted to remain planar. These angles are approximate, since there is some distortion within the two planes intersecting at the N5—N10 axis that renders a measure of the bending angle somewhat ambiguous, but their magnitudes are quite reasonable in light of the experimental evidence from other flavin derivatives. The acetyl carbonyl is predicted to lie in the plane of the isoalloxazine ring for the three oxidized isomers and for the reduced 7α-keto. The acetyl carbonyl is rotated 90° out of plane in the 6 and 8α-keto isomers.

The structures of the enol tautomers, for which there is no experimental comparison, are generally predicted to be more planar than the keto structures. Only the reduced 8-acetyl isomer is predicted to be bent, by 14°, and the other two are

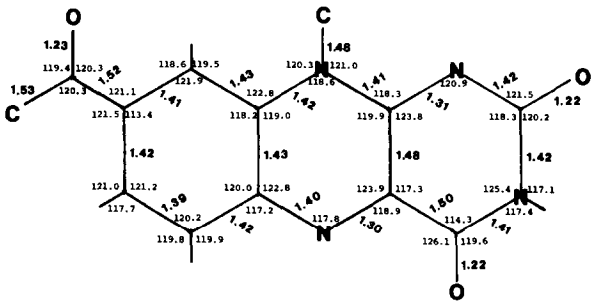


FIG. 3. MNDO optimized bond angles (degrees) and bond lengths (Å) of (oxidized) 8-acetyl-10-methylisoalloxazine.

TABLE 2

MNDO CALCULATED HEATS OF FORMATION, DIPOLE MOMENTS, AND FOLDING ANGLES, ϕ, OF x-ACETYL-10-METHYLISOALLOXAZINES

Compound	H_f (kcal/mol)	μ (Debye)	ϕ (Degrees)
Oxidized:			
8-	−35.90	5.84	0
7-	−37.20	7.24	0
6-	−27.66	9.54	0
Reduced (α-keto):			
8-	−58.57	1.98	30
7-	−44.09	7.55	0
6-	−56.32	5.28	25
Reduced (α-enol):			
8-	−44.45	7.92	14
7-	−14.67	5.68	0
6-	−36.11	5.80	0

planar. The more planar nature of the enols correlates with the increased π-bonding at the C9a—N10 and N10—C10a bonds in the central ring (Table 1).

The heats of formation (Table 2) of the tautomeric species calculated from the MNDO method predict for all three isomers that the keto tautomer should greatly dominate the equilibrium, the ratio being greatest for the 7-acetyl isomer and least for the 8-acetyl isomer. This qualitative order of relative stabilities seems reasonable since it is not possible to write a valence structure for the least favored 7-acetyl tautomer without a formal charge separation. The most reasonable transfer is between N5 and N10, making the former more negative and the latter more positive. Such a relative charge separation between N5 and N10 is corroborated by comparison of the INDO net atomic charges obtained for the 8- and 7-acetyl

TABLE 3

INDO NET ATOMIC CHARGES

	8-Acetyl-10-methylisoalloxazine			7-Acetyl-10-methylisoalloxazine
	Oxidized	Reduced	α-Enol	α-Enol
N1	−0.435	−0.309	0.096	−0.328
C4a	0.211	0.083	0.096	0.196
N5	−0.292	−0.345	−0.374	−0.406
N5a	0.122	0.114	0.192	0.127
C9a	0.131	0.100	0.105	0.150
N10	−0.223	−0.308	−0.286	−0.151
C10a	0.359	0.259	0.271	0.147
C(acyl)	0.403	0.421	0.194	0.165
O(acyl)	−0.584	−0.588	−0.472	−0.467

enols (Table 3). The charge on N5 becomes more negative by 0.03 and on N10 more positive by 0.14 electrons.

While the predicted trends in tautomer stabilities are reasonable, there are, nevertheless, two reasons to question the predicted balance of tautomeric equilibrium within a particular isomer: (1) MNDO studies of keto–enol tautomers in related aromatic systems have encountered problems in predicting relative tautomer stabilities. Specifically, MNDO has incorrectly predicted the balance of tautomeric equilibria in uracil (*20, 21*) and hydroxypyridine (*22*). In these instances MNDO apparently overestimates the stability of tautomers that contain an aromatic ring. (2) A further consideration is that the MNDO calculation does not account for solvent effects such as hydrogen bonding with the aqueous solvent. A very crude estimate of the effect of relative solvation energies on tautomer equilibria can be obtained from the expression, due to Onsager, for the energy of a spherical dipole interacting with a continuous dielectric medium (*23a,b*) Such a model predicts a modest stabilization of several kcal/mole for the 8α-enol tautomer relative to the keto, and a negligible effect for the 6- and 7-acetyl compounds (*24*). In light of these considerations, theoretical predictions of spectra, rather than relative energies, should be a more reliable criterion for identifying the nature of the reduced species.

Spectra. Table 4 gives the transition energies, intensities, and assignments obtained from the INDO/S calculation, along with the experimental data. For the oxidized species, good agreement is obtained between calculated and experimental spectra. The analogs are predicted to have similar spectra, which is consistent with the close similarity of the experimental specta. As shown in Figs. 4a and b, when the systematic blue shift obtained in the calculated frequencies are taken into account, the similarity between calculated and observed spectra of the oxidized species is even more apparent. It is interesting to note that the energies and

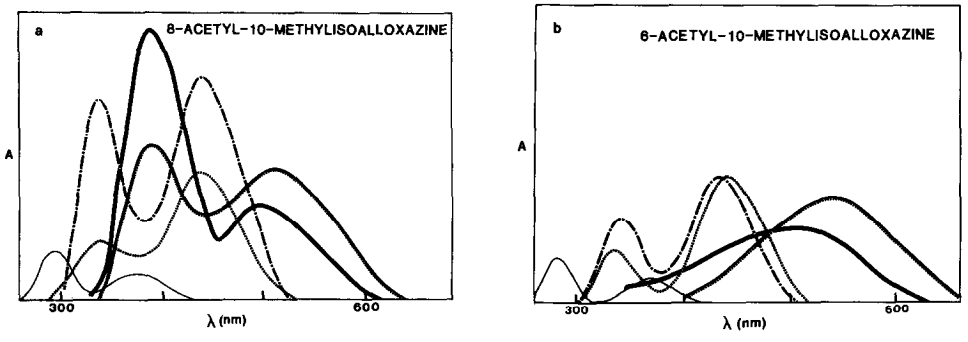


FIG. 4. Comparison of observed (Ref. (*1c*)) and predicted spectra for (a) 8-acetylisoalloxazine and (b) 6-acetylisoalloxazine: (—) observed reduced spectrum, (‾‾) fit calculated for reduced α-enol, (—) fit calculated for reduced α-keto tautomer, (– · – ·) observed oxidized spectrum, (· · ·) fit calculated from theoretical predictions for oxidized form. Fitted curves were obtained by subtracting from the theoretical energies in Table 4 a small offset that corresponds to the average difference of the theoretical and experimental transition energies of the oxidized species. A line transition with the theoretical intensity at the adjusted energy is then folded with the average bandwidth of the experimental spectrum.

TABLE 4

SPECTRA OF *x*-ACETYL-10-METHYLISOALLOXAZINES

	Experimental[a]		Calculation		
	E(kk)	Osc.str.	E(kk)	Osc.str.	Assignment[e]
Oxidized:					
8-	22.8	0.33	27.1	0.53	fl[b] → fl*
	29.6	0.45	33.7	0.33	ph[c] → fl*
	36.6	(sh)	37.5	0.32	fl → (fl,acetyl)*
6-	23.1	0.32	26.7	0.55	fl → (fl,C=O(12))*
	29.2	0.20	33.7	0.23	ph → (fl,C=O(12))*
	—	—	37.7	0.09	fl → (fl,acetyl)*
	37.9	0.88	39.3	0.61	fl → fl*
7-	23.2	0.32	28.1	0.57	fl → fl*
	29.0	0.15	33.9	0.10	ph → fl*
			37.7	0.95	fl → (fl,acetyl)*
Reduced (α-keto):					
8-			31.0	0.12	fl → C=O(12)*
			38.3	0.21	fl → C=O(11)*
			39.3	0.12	fl → fl*[f]
			41.7	0.22	fl → C=O(11)*
			44.3	0.22	ph → C=O(12)*
6-			31.2	0.11	fl → C=O(12)*
			39.4	0.21	fl → C=O(11)*
			42.6	0.26	fl → C=O(11)*
			44.2	0.24	fl → ph*
7-[d]	21.3	0.02	30.6	0.20	fl → (fl,C=O(12),acetyl)*
			33.1	0.28	fl → C=O(11)
			37.7	0.95	fl → (ph,acetyl)*
Reduced (α-enol):					
8-	20.2	0.13	23.8	0.59	fl → (fl,α-OH)*
	25.6	0.40	29.9	0.63	fl → C=O(12)*
	32.2		36.6	0.15	ph → (fl,α-OH)*
	35.7	(sh)	38.7	0.23	fl → (fl,α-OH)*
	40.0		48.3	0.24	ph → C=O(12)
6-	19.6	0.18	22.3	0.47	(fl,α-OH) → (fl,α-OH)*
	—	—	25.4	0.21	(fl,α-OH) → C=O(12)*
	33.1		39.3	0.11	fl → (fl,α-OH)*
	39.7	1.30	42.2	0.55	ph → (fl,α-OH)*
7-			8.2	0.18	fl → fl*
			22.5	0.47	fl → C=O(12)*
			26.2	0.28	fl → C=O(11)*
			30.6	0.14	fl → ph*
			32.3	0.57	ph → fl*
			36.2	0.87	ph → fl*

[a] From Ref. (*1c*) unless otherwise noted.
[b] Flavinoid.
[c] Benzenoid.
[d] E. T. Kaiser, private communication.
[e] $\pi \rightarrow \pi^*$ MO promotions, except where indicated.
[f] $\pi \rightarrow \sigma^*$.

intensities calculated for oxidized lumiflavin in Ref. (*14*) compare quite closely to those for the oxidized acetylflavin analogs in Table 4.

The calculated spectra allow assignment of the observed transitions in the oxidized species. The lowest transition in the oxidized derivatives is assigned to a HOMO → LUMO$\pi\pi^*$ transition where both molecular orbitals are delocalized over the fused rings of the isoalloxazine nucleus and have negligible contributions from orbitals on the acetyl moiety. The next transition arises out of a largely benzenoid-ring localized πMO. Only the next higher transition in each oxidized species contains some direct acetyl participation.

The spectra of the reduced species show much more variability between isomers; the bleached spectrum of the 7-acetyl derivative differs qualitatively from the robust spectra of the 6- and 8-acetyl isomers (Fig. 5). The featureless spectrum of the reduced 7-acetyl species is characteristic of a planar 1,5-dihydroflavin, which would be consistent with the geometry calculated for this species. As noted above, the reduced forms of the 6- and 8-acetylisoalloxazines are observed to have anomalous red-shifted absorptions of moderate to strong intensity. The calculated spectra (Table 4 and Figs. 4a and b) of the keto tautomers of each isomer show no transitions that could be correlated with such a red-shifted absorption. The transitions of the keto tautomers are all calculated to occur above 30,000 cm^{-1} and are assigned to flavin → isoalloxazine carbonyl $\pi\pi^*$ MO transitions. The enol tautomers, on the other hand, are indeed predicted to have a strongly red-shifted absorption. These transitions in the 6- and 8-acetyl derivatives contain significant hydroxyl involvement and are qualitatively different from the lowest energy transition found in the 1,5-dihydroisoalloxazines. This very close correspondence between the spectra observed for the reduced 6- and 8-acetylisoalloxazines and the spectra calculated for their corresponding enols (Fig. 4) strongly suggests that α-enol tautomers are responsible for the observed spectra.

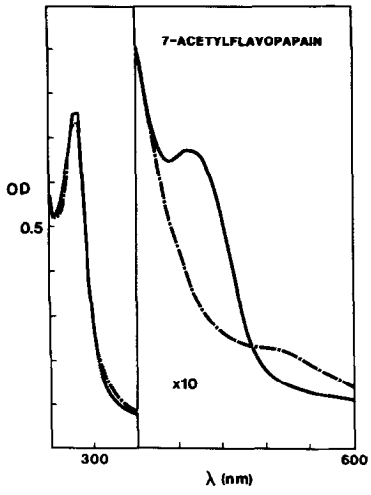


FIG. 5. Experimental spectrum of 7-acetylflavopapain (Ref. (*25*)). (—) Oxidized flavin, (– · – · –) reduced flavin.

CONCLUSIONS

It is expected that the enol-to-keto ratio is more favorable in the 6- and 8-acetyl reduced flavin analogs than it is in the 7-acetyl analog for observing the red-shifted absorption peak indicative of the enol. There is in fact a close correspondence between the observed spectra for the 6- and 8-acetylisoalloxazine reduction products and the predicted spectra for the 6α- and 8α-enols. This correspondence strongly supports the assignment of the latter species as the reduction products. Similarly, the observed spectra of the reduced 7-acetyl flavin analogs, reduced 7-acetyl-10-methylisoalloxazine and 7-acetylflavopapain are assigned as originating from the α-keto tautomers, i.e., the 1,5-dihydro reduction products, on the basis of correspondence with calculated spectra; assignments which are also consistent with the expected trend in tautomer stabilities.

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