Carbon-13 Nuclear Magnetic Resonance Study on the Dynamics of the Conformation of Reduced Flavin†

Chrit T. W. Moonen, Jacques Vervoort, and Franz Müller*

ABSTRACT: Several flavin model compounds in the reduced state have been investigated by ^{13}C NMR techniques. The NMR spectra were recorded in dependence of temperature, in the range of 30 to −100 °C. The results show that the activation barrier for the ring inversion ("butterfly" motion) is too low to be observed directly. In order to be able to detect the barrier of the ring inversion, it was coupled with a side-chain rotation. In this way, the intrinsic barrier for the ring inversion is increased by the barrier of the side-chain rotation, which allowed detection of the former barrier. It is shown that the intrinsic barrier for the ring inversion is <20 kJ/mol. Moreover, it is shown that previous results of Tauscher et al. [Tauscher, L., Ghisla, S., & Hemmerich, P. (1973) *Helv. Chim. Acta 56*, 630–649] are incorrect and nitrogen inversion is not observed. Symmetry arguments in the dynamic processes are discussed. From the low activation barrier for the ring inversion, it can be concluded that the conformation of the reduced flavin can be easily influenced upon binding to apoflavoproteins. This aspect might be of importance in the regulation of the function of the flavin prosthetic group in biological systems.

Reduced 1,5-dihydroflavin plays an important role in biological reactions catalyzed by flavoproteins. It is known from crystallographic studies (Kierkegaard et al., 1971) on flavin model compounds that reduced flavin possesses a bent conformation, often referred to as "butterfly" conformation. It has been proposed that the activation barrier for the transition from the bent to the planar conformation provides a means of regulating the redox potential of protein-bound flavin (Simondsen & Tollin, 1980; Tauscher et al., 1973; Van Schagen & Müller, 1981). In this concept, the apoflavoprotein forces the bound flavin to attain a certain conformation. This concept seems to be supported by theoretical calculations indicating that an activation barrier of 145 kJ/mol exists between the bent and the planar conformations (Palmer & Platenkamp, 1979). Tauscher et al. (1973) investigated the nitrogen inversion in reduced flavin by ^{1}H NMR.[1] An activation barrier of about 42 kJ/mol for the inversion of the N(5) atom was calculated from these results. This seems to be in contradiction with our recent ^{13}C and ^{15}N NMR data (Moonen et al., 1984). These results demonstrate that the conformation of reduced flavin is mainly governed by intramolecular overcrowding effects [substitution at N(1) and N(5)] and the polarity of the solvent. The ease in which the endocyclic angle of the N(5) and the N(10) atom can be modulated independently is especially striking. This suggests that the activation barrier for reduced flavin to attain a planar conformation must be low.

Preliminary ^{13}C NMR results (Van Schagen & Müller, 1980) strongly indicated that this technique is particularly useful for investigation of the dynamics of the conformational changes of reduced flavin. Since the ^{13}C chemical shifts of flavin are mainly governed by the π-electron density at the carbon atoms under study (Van Schagen & Müller, 1980), ^{13}C NMR should yield a much better insight into the dynamics of the conformational change than ^{1}H NMR, where only a limited number of nuclei in flavin are available. Furthermore, the resolution of ^{13}C NMR spectra at high field strength is much better than the splitting of a single proton resonance line to an AB pattern, as used by Tauscher et al. (1973). Our results demonstrate that the temperature-dependent ^{13}C NMR spectra of reduced flavin yield detailed information on the conformation of reduced flavin and allow calculation of a more accurate value for the activation barrier. This study also shows that the nitrogen inversion is not observed and that the spectral changes are related to the butterfly motion of the molecule. This is in contrast to published results (Tauscher et al., 1973).

Materials and Methods

The compounds used in this study were prepared according to published procedures: 1,3,7,8,10-pentamethyl-5-acetyl-1,5-dihydroisoalloxazine, 1,3,7,8,10-pentamethyl-5-benzyl-1,5-dihydroisoalloxazine, 1,3,5,7,8,10-hexamethyl-1,5-dihydroisoalloxazine, and 1,3,7,8-tetramethyl-5-acetyl-5,10-dihydroalloxazine, (Dudley & Hemmerich, 1967); 3,7,8-trimethyl-1,10-ethano-5-acetyl-1,5-dihydroisoalloxazine (Müller & Massey, 1969); the parent compound from riboflavin (Grande et al., 1977); 3-methyl-5-acetyl-1,5-dihydrotetraacetylriboflavin (Van Schagen & Müller, 1980); 3-methyl-1,5-dihydrotetraacetylriboflavin directly in the NMR tube by a two-phase reduction (Van Schagen & Müller, 1980). Subsequently, the aqueous phase was removed by means of a syringe under anaerobic conditions. The concentration of the flavin solutions was 30–50 mM.

Wilmad 10-mm precision tubes were used. ^{13}C NMR measurements were performed at 75.5 MHz on a Bruker 300 CXP spectrometer. Broad-band decoupling (3 W) was applied. A repetition time of 1 s was used. Pulse width was 30°. A total of 5000 transients was usually accumulated. Conversion rates were determined from the coalescence points according to the method of Binsch (1975). Spectra exhibiting unequal populations of the various forms were analyzed by the method of Shanan-Atidi & Bar-Eli (1970). Activation barriers were determined from the Eyring equation as described by Binsch (1975).

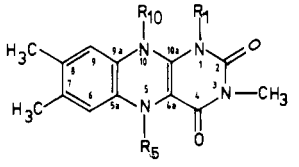
Results and Discussion

As has been discussed previously by Tauscher et al. (1973), the following dynamic processes have to be considered in the analysis of temperature-dependent conformations of reduced

† From the Department of Biochemistry, Agricultural University, De Dreijen 11, 6703 BC Wageningen, The Netherlands. *Received January 17, 1984.* This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

[1] Abbreviations: NMR, nuclear magnetic resonance; $MeTARFH_2$, 3-methyl-1,5-dihydrotetraacetylriboflavin.

0006-2960/84/0423-4868$01.50/0



1: $R_1R_{10}=CH_2-CH_2, R_5=COCH_3$
2: $R_1=R_{10}=CH_3, R_5=COCH_3$
3: $R_1=CH_3, R_{10}=H, R_5=COCH_3$
4: $R_1=H, R_{10}=$ tetraacetylribityl, $R_5=COCH_3$
5: $R_1=R_5=H, R_{10}=$ tetraacetylribityl
6: $R_1=R_{10}=CH_3, R_5=C_7H_7$
7: $R_1=R_5=R_{10}=CH_3$

FIGURE 1: Structures of the compounds investigated.

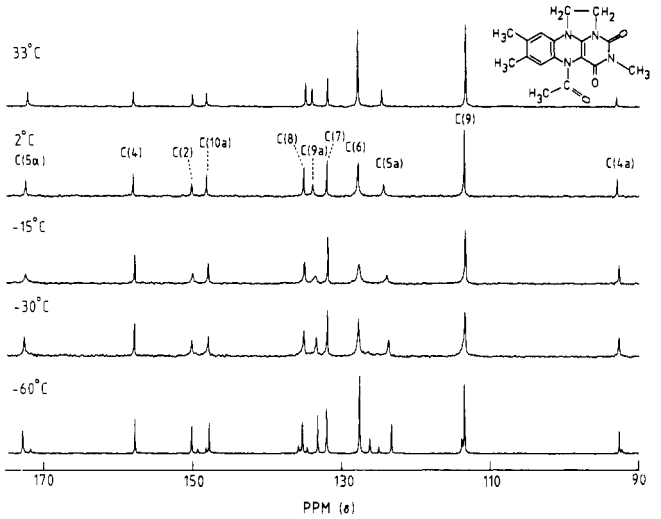


FIGURE 2: Temperature dependence of the natural abundance ^{13}C NMR spectrum of 3,7,8-trimethyl-1,10-ethano-5-acetyl-1,5-dihydroisoalloxazine (**1**) in a mixture of chloroform/methanol (20:1 by volume).

flavin: (1) the rotation of the substituent at N(5) around the N(5)–N(5α) bond; (2) the inversion of the N(5) and the N(10) atoms; (3) the butterfly motion of the reduced flavin molecule, i.e., the flipping around the N(5)–N(10) axis. It is often difficult to discriminate experimentally between nitrogen inversion and ring inversion (butterfly motion) in heterocycles (Lambert et al., 1971). Some certainty with regard to this problem can be obtained by using various model compounds, as will be shown.

The structures of the compounds investigated in this study are shown in Figure 1. Whereas compounds **5–7** show no splitting, not even at −60 °C, compounds **1–4** exhibit splitting of the resonance lines upon cooling. The dependence on the temperature of the ^{13}C NMR spectra of compounds **1** and **2** is shown in Figures 2 and 3, respectively. At low temperature, both compounds give rise to splitting of all or almost all resonances, although the population stoichiometry of the splitting is different (Table I). The activation barrier calculated from the ^{13}C NMR spectra for several compounds is summarized in Table II. The question now arises which dynamic process causes the observed splitting of the resonances. The splitting patterns shown in Figures 2 and 3 reflect a dynamic process that influences the electronic structure of the whole isoalloxazine ring as almost all resonances are split. A possible freeze of the acetyl rotation would populate two rotamers due to steric hindrance with the C(4) carbonyl function and the N(10) substituent, i.e., the structure shown in Figure 4A and its rotamer where the acetyl group is inverted by 180°. It is not expected that the two rotamers would influence the electronic structure of flavin very differently. Moreover, the

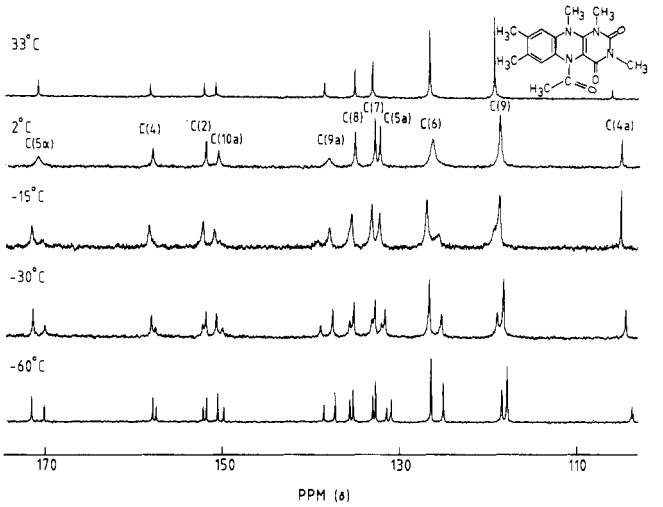


FIGURE 3: Temperature dependence of the natural abundance ^{13}C NMR spectrum of 1,3,7,8,10-pentamethyl-5-acetyl-1,5-dihydroisoalloxazine (**2**) in a mixture of chloroform/methanol (20:1 by volume).

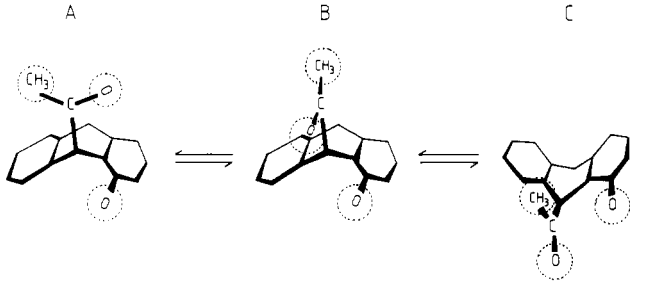


FIGURE 4: Schematic representation of the butterfly motion (ring inversion) and the rotation of the N(5) acetyl side chain. The configuration of the acetyl side chain in A is favored as the steric overcrowding effect with the carbonyl group at position 4 is minimized [also the para-overcrowding effect with the N(10) substituent is minimized]. To allow the ring inversion (butterfly motion) A → C, it is necessary that the acetyl side chain moves in such a way (A → B) that the peri-overcrowding effect with the C(4) carbonyl group is minimized during the planar transition state (see also text).

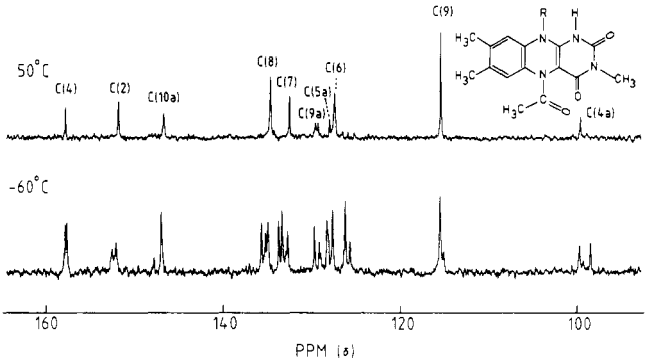


FIGURE 5: Natural abundance ^{13}C NMR spectra of 3-methyl-5-acetyl-1,5-dihydrotetraacetylriboflavin (**4**) in a mixture of chloroform/methanol (20:1 by volume).

splitting and population stoichiometry of the resonances are specific for each compound (cf. Table I). For example, most resonances of compound **4** are split into three lines (Figure 5; for a discussion, see below). It can therefore safely be concluded that the splitting of the resonance lines is not caused by a freeze of the acetyl rotation.

This effect could be caused by inversion of one of the nitrogen atoms [N(5) or N(10)], by the so-called butterfly motion (i.e., ring inversion), or by a combination of effects, both resulting in a change of the nitrogen substituents from axial to equatorial position and vice versa. An inversion of

Table I: Temperature Dependence of Relevant ^{13}C Chemical Shifts of Some N(5)-Substituted Reduced Free Flavins in Different Solvents

	^{13}C chemical shifts (ppm) of compound									
	1[a]		**2**[a]		**3**[a]		**4**[a,b]		**2**[c]	**2**[d]
atom	33 °C	−60 °C	33 °C	−60 °C	33 °C	−60 °C	50 °C	−60 °C	−60 °C	−60 °C
C(2)	150.30	150.35	152.04	152.18	150.39	150.64	152.18	152.58	152.23	152.80
		149.51		151.82[e]		150.15[e]		152.14	151.89[e]	152.58[e]
C(4)	158.24	157.99	158.17	157.95[e]	157.60	157.75	158.16	157.85	158.14[e]	158.63[e]
				157.55		157.41[e]		157.65		157.72
C(4a)	93.19	92.70[e]	106.01	103.75[e]	95.30	94.19[e]	99.93	99.71	103.02[e]	103.02[e]
		92.31		103.60		93.46		98.51	102.67	102.65
C(5a)	124.86	125.10	133.08	131.45	125.74	125.13[e]	128.30	128.24	130.79	132.48
		123.43[e]		130.91[e]		124.61		127.63	130.17[e]	131.91[e]
C(6)	128.14	127.75[e]	126.65	126.42[e]	127.01	126.72[e]	127.70	126.12	126.18[e]	126.69[e]
		126.38		125.08		126.55		125.69	124.88	125.47
C(7)	132.16	132.16[e]	133.12	133.02	132.50	132.01[e]	132.85	133.73	133.38	132.99
				132.67[e]		131.89		133.34	132.82[e]	132.01[e]
								132.70		
C(8)	135.15	135.98	135.10	135.62	135.25	135.74	134.98	135.64	136.06	135.84
		135.49[e]		135.25[e]		135.17[e]		135.20	135.71[e]	135.21[e]
								134.91		
C(9)	113.63	113.97	119.34	118.46	116.57	116.99	115.71	115.47	118.26	119.37
		113.63[e]		117.82[e]		116.57[e]		115.08	117.77[e]	118.61[e]
C(9a)	134.27	134.81	138.56	138.58	132.50	132.99[e]	129.60	129.71	138.29	140.17
		133.43[e]		137.31[e]		132.48		129.17	137.14[e]	139.07[e]
C(10a)	148.39	148.39	150.71	150.52[e]	148.14	148.70[e]	147.06	147.82	150.89[e]	151.77[e]
		147.99[e]		149.83		148.14		147.01	150.42	150.74
C(5α)	172.46	173.14[e]	170.79	171.72[e]	172.36	173.14	169.34	169.71	172.41[e]	171.32[e]
		172.06		170.25		171.60		169.46	170.98	169.64

[a] Solvent is C^2HCl_3/CH_3OH (20:1 by volume). [b] Only the two highest peaks are listed (see Figure 5). [c] Solvent is C^2HCl_3/CH_3OH (7:3 by volume). [d] Solvent is deuterated acetone. [e] Highest populated.

the N(10) atom can be excluded since the gross effects observed for compounds **1** and **2** are very similar, with almost equal activation barriers. As has been shown by Moonen et al. (1984), compound **1** contains an sp^2-hybridized N(10) atom, whereas the N(10) atom in compound **2** exhibits considerable sp^3 character. Nitrogen inversion is strongly dependent on the endocyclic angle and consequently on the hybridization of the nitrogen atom (Lehn & Wagner, 1970; Lambert et al., 1971).

Although the hybridization of the N(10) atom differs in compounds **1** and **2**, very similar effects are observed for both compounds. We therefore conclude that the splitting patterns are not caused by N(10) inversion. This leaves us with the dynamic process of the N(5) inversion and/or the butterfly motion. Whichever process predominates, a partial rotation of the acetyl group is needed, as demonstrated in Figure 4. The acetyl function has to align along the N(5)–N(10) axis before ring inversion or N(5) inversion can take place. Due to considerable overlap between the carbonyl function at C(4) and the acetyl group at N(5), this leads to an energetically rather unfavorable situation. The foregoing analysis shows that the introduction of the acetyl group at N(5) leads to a coupling (to some degree) of the acetyl rotation with the N(5) and/or ring inversion. The coupling of the two dynamic processes is evident from the observation that no splitting patterns could be observed for compounds **5**–**7**. The activation barrier of the acetyl rotation can be roughly estimated from published data on amino and amide rotation [for a review, see Binsch (1975)]. A rotation barrier of 39.5 kJ/mol was determined, for example, for Trimethyl(dichloroamino)methane, containing an sp^3 hybridized nitrogen atom (Bushweller et al., 1973). In amides, possessing a partial double bond, the barrier was found to be about 60 kJ/mol if only a small steric hindrance is present in the molecule, and the barriers become much higher if steric hindrance is increased (Binsch, 1975).

The activation barrier for the acetyl rotation in flavin is estimated to be at least 60 kJ/mol. Tauscher et al. (1973) suggested that ring inversion in flavin is energetically the most favored process. This suggestion is in agreement with published data obtained on other heterocyclic compounds. For instance, Lehn & Wagner (1970) and Lambert et al. (1971) showed that the activation barrier for nitrogen inversion in cyclic compounds ranges from 40 kJ/mol to much higher values for sp^3-hybridized nitrogen atoms not containing a covalently bound hydrogen atom. On the other hand, ring inversion in cyclohexane shows an activation barrier of 42 kJ/mol (Anet & Anet, 1975). Introduction of a double bond into this system, however, markedly decreases the activation barrier (Anet & Anet, 1975). Bernard & St-Jacques (1973) determined a barrier of 20–25 kJ/mol in cyclohexene. The drastically decreased barrier was ascribed to the fact that cyclohexene cannot adopt the low-energy chair conformation. This means that the more planar the conformation of a molecule becomes the lower the barrier will be, as has also been demonstrated for piperidine and derivatives thereof, containing a sp^2 center (Gerig, 1968; Jensen & Beck, 1968; Lambert & Oliver, 1968). The barriers determined for cyclohexane, cyclohexene, and the piperidine derivatives are of about the same magnitude. This also strongly indicates that the replacement of a carbon atom in cyclohexane by a nitrogen atom does not influence the activation barrier much. This suggests that the properties of the pyrazine subnucleus of reduced flavin are compatible with those of simple cyclohexane and cyclohexene derivatives. Thus, the actual activation barrier for ring inversion must indeed be far below 60 kJ/mol, and the apparent high activation barriers reported in this study are caused by the high barrier for the acetyl rotation.

From this it can also be concluded that ring inversion in reduced flavin is considerably more favored than inversion of the acetylated N(5) atom. This implies that we are observing the effects of the ring inversion in Figures 2 and 3, although the calculated activation barriers from the splitting patterns represent its activation energy increased by the barrier of the unfavorable acetyl rotation.

The splitting patterns of all N(5)-acetylated flavins investigated are rather similar, and the activation barriers hardly

Table II: Activation Barriers in Chloroform Calculated from Temperature-Dependent ^{13}C NMR Spectra by the Method of Shanan-Otidi & Bar-Eli (1970)

compd	activation barrier, ΔG^* (kJ/mol), at the coalescence temp	coalescence temp (K)
1	55.2	258
2	55.6	273
3	57.6	273
4	61.9	306
5	*a*	
6	*a*	
7	*a*	

[a] No splitting observed upon cooling to 213 K.

differ (Table II). This indicates that the activation barrier for the ring inversion is hardly dependent on the N(10) substituent (Table II). Although a change in the size of the N(10) substituent undoubtedly influences the activation barrier for the ring inversion, we observe a negligible influence of the N(10) substituent on the activation barrier of the coupled dynamic process (Tables I and II). This clearly demonstrates that the activation barrier for the coupled process is mainly caused by the barrier for the acetyl rotation only. As a consequence, the barrier for the ring inversion must be very low.

This interpretation is in full agreement with earlier observations (Moonen et al., 1984) showing that the degree of hybridization of the N(5) and the N(10) atoms in reduced flavin can easily be influenced by substitution. Theoretical calculations by Dixon et al. (1979) resulted in an activation barrier of 16 kJ/mol for the ring inversion of unsubstituted 1,5-dihydroflavin. In our opinion, this value is, also considering the published results on cyclohexane, cyclohexene, and piperidine derivatives, close to the actual activation barrier. In contrast, the theoretical value of 145 kJ/mol for the ring inversion of reduced flavin, calculated by Palmer & Platenkamp (1979), must be incorrect.

The interpretation of our results is in contradiction to work published by Tauscher et al. (1973). These authors investigated ^1H NMR spectra of N(5)-alkylated flavin derivatives in dependence of temperature and ascribed the observed effects to the inversion of the N(5) atom. They excluded a rotation of the N(5) side chain around the N(5)–C(5α) or C(5α)–C(5β) axis. This conclusion was based on the fact that 1,3,7,8-tetramethyl-10-benzyl-5-acetyl-1,5-dihydroisoalloxazine did not show a splitting of the methyl group of the N(5) acetyl group, not even at −107 °C. It is, however, by no means certain that the two possible rotamers can be resolved by ^1H NMR. Even if the two rotamers could be resolved, their detection could well be obscured by a large population difference. Furthermore, the maximum activation energy for the acetyl rotation, derived by Tauscher et al. (1973) from the absence of a splitting in the ^1H NMR spectrum of 1,3,7,8-tetramethyl-10-benzyl-5-acetyl-1,5-dihydroisoalloxazine, is far below what would be expected for such a rotation [cf. Binsch (1975)], taking into consideration (a) the partial double bond character in the amide-like N(5)–C(5α) bond, (b) the peri-overcrowding with the carbonyl function at C(4), and (c) the para-overcrowding with the large N(10) benzyl substituent.

Moreover, it can be concluded directly from the published spectra of Tauscher et al. (1973) that the interpretation of their data is incorrect. If it were true that rotation of the N(5) side chain in N(5)-benzylated flavin is fast on the NMR timescale down to −100 °C, then the methylene protons of the benzyl side chain should remain equivalent because of this fast rotation. Consequently, freezing the inversion of the N(5) atom would lead to a splitting of the singlet due to the methylene group into a doublet of probably unequal population. The actual observed pattern of a quartet (two doublets of equal population) by Tauscher et al. (1973) must be due to the fact that the rotation of the benzyl group is frozen, rendering the methylene protons unequivalent, resulting in a multiplet of an AA′BB′ pattern. The published value for the barrier of 42.4 kJ/mol (Tauscher et al., 1973) is also in the range expected for the side-chain rotation (Binsch, 1975). Moreover, we could not detect any splitting or even a broadening in the ^{13}C NMR spectra of compound **6** in acetone down to −95 °C. The apparent discrepancy between the ^1H and ^{13}C NMR results lies in the fact that freezing of the rotation of the benzyl group will only be observable in the ^{13}C NMR spectrum if it affects the chemical shift of the carbon atom of the methylene group. This is obviously not the case.

It is desirable to briefly discuss the population differences observed at low temperature for the compounds investigated. Ring inversion changes the stereochemical position of the N(5) and N(10) substituents from axial to equatorial position and vice versa. It is evident from Figure 4 that the axial position of the N(5) substituent is favored. Nevertheless, the population stoichiometry (Table I) reaches almost 1:1 for compound **4** in chloroform and for **2** in the mixed solvent $CHCl_3/CH_3OH$ (7/3) (Table I). This indicates that the unfavored equatorial position of the N(5) acetyl group must be accompanied by another effect in these compounds. This effect most probably originates from a steric hindrance exerted on the N(10) substituent. In compound **2**, for example, the axial position of the N(10) methyl group is favored above the equatorial position due to the interaction with the N(1) methyl group. Thus, the fact that we do not observe a large population difference for the two frozen conformations of **2** in chloroform can only be explained by assuming that the N(5) substituent is in axial position; then the N(10) substituent is in equatorial position and vice versa. This notion is important to understand the solvent-dependent population differences for compound **2** (Table I). In the accompanying paper (Moonen et al., 1984), we have shown that in a protic solvent the partial sp^2 character of the N(10) atom is increased due to π-electron delocalization to O(2α) and O(4α), whereas the hybridization of N(5) is relatively unchanged. The consequence of a more hybridized N(10) is that the steric hindrance between the N(10) substituent in equatorial position and the N(1) substituent is increased. This conformation [i.e., the N(5) acetyl group in axial and the N(10) methyl group in equatorial position] of the isoalloxazine ring is therefore relatively unfavored as compared to the second conformation [N(5) acetyl group in equatorial and the N(10) methyl group in axial position]. Therefore, the population stoichiometry goes in the direction of 1:1 if the solvent becomes more protic. The fact that N(10) becomes more planar if more methanol is added can be easily verified by the upfield shift of C(4a) and C(5a), as outlined in the accompanying paper. It should be noted that Tauscher et al. (1973) used acetone as the solvent in most experiments. It can be seen from Table I that in this solvent a large population difference exists, which might well obscure a detection by ^1H NMR as used by Tauscher et al. (1973). Moreover, we wish to emphasize that the terms axial and equatorial position for reduced flavin should be regarded with caution, because of the partial sp^2 character of the N(10) and N(5) atoms.

The temperature dependence of compound **4** is shown in Figure 5. It is evident that some resonances are split into three lines. This indicates that three structures are well populated. This compound contains a bulky side chain at N(10). As the

N(10) is rather sp^2 hybridized (cf. the accompanying paper), N(10) inversion cannot be fully excluded. Therefore, the spectrum obtained at −60 °C (Figure 5) can be explained as follows: (1) the N(5) substituent in axial and the N(10) substituent in equatorial position; (2) the N(5) substituent in axial and the N(10) substituent in axial position; (3) the N(5) substituent in equatorial and the N(10) substituent in axial position.

The symmetry aspects of the dynamics of nitrogen inversion and ring inversion will be discussed briefly because these aspects are important for a thorough interpretation of the data (Lambert et al., 1971). Thus, if the ring inversion (butterfly motion) in reduced flavin were followed immediately by nitrogen inversion of the N(5) and the N(10) atoms, the mirror image of the initial conformation would be formed, resulting in identical NMR spectra. Consequently, the ring inversion in reduced flavin can only be observed in flavin derivatives in which the barrier for nitrogen inversion is higher than that for the ring inversion. In compounds **1**–**4** for instance, the ring inversion could only be studied because of the presence of the N(5) acetyl group. In the absence of the acetyl group, the N(5) inversion follows the ring inversion (due to fast proton tunneling), yielding the mirror image of the initial state in agreement with the experimental observation that NMR spectra of N(5)-unsubstituted flavins show no dependence on temperature. The ring inversion of free 1,5-dihydroflavin, involved in biological reactions, can therefore only be studied by introduction of sterical hindrance at the N(5) atom since the N(10) atom will probably have a low inversion barrier, owing to its partial sp^2 character. It follows from this that it will be rather difficult to design experiments where the two coupled dynamic processes can be studied independently by ^{13}C and ^{1}H NMR. With respect to the study of the N(5) inversion by Tauscher et al. (1973), it must be mentioned that these authors did not consider symmetry arguments in the interpretation of their experimental results. Thus, if effects of N(5) inversion have been observed, they have been caused either by a direct N(5) inversion or by ring inversion followed by N(10) inversion resulting in the enantiomeric form, which cannot be distinguished from the initial form by conventional NMR methods. The energetically favored pathway will of course be dominant. This means that the N(5) inversion will not be observable if the other pathway is energetically favored as is the case in compound **6**, investigated by Tauscher et al. (1973).

The existence of mirror images of flavin has some implications for studies on the binding of flavin and flavin analogues by apoflavoproteins. Since, as shown previously (Moonen et al., 1984), the N(10) atom in oxidized flavin lies somewhat out of the molecular plane, an enantiomer also exists in this state. If the apoflavoprotein binds the flavin molecule stereospecifically, as can be expected, then half the amount of an added stoichiometric concentration of flavin with respect to the apoflavoprotein will be bound faster by the apoprotein than the other half, because some time will be required for the conversion into the other enantiomeric form. However, as the conversion will occur with a high rate (low activation barrier), this aspect will probably only be of importance in kinetic studies using techniques of high time resolution. A rough calculation shows that the time resolution needed to discriminate between the two enantiomers would be in the order of 0.1 ns at 27 °C and 0.2 ns at 0 °C.

Acknowledgments

We are indebted to J. C. Toppenberg-Fang for typing the manuscript, to M. M. Bouwmans for the preparation of the figures, and to Dr. C. A. H. Rasmussen for carefully reading the manuscript and the preparation of some compounds.

References

Anet, F. A. L., & Anet, R. (1975) in *Dynamic Nuclear Magnetic Resonance Spectroscopy* (Jackman, L. M., & Cotton, F. A., Eds.) pp 543–619, Academic Press, New York.

Bernard, M., & St-Jacques, M. (1973) *Tetrahedron 29*, 2539–2544.

Binsch, G. (1975) in *Dynamic Nuclear Magnetic Resonance Spectroscopy* (Jackman, L. M., & Cotton, F. A., Eds.) pp 45–81, Academic Press, New York.

Bushweller, C. H., Anderson, W. G., O'Neill, J. W., & Bilofsky, H. S. (1973) *Tetrahedron Lett.*, 717–720.

Dixon, D. A., Lindler, D. L., Branchard, B., & Lipscomb, W. N. (1979) *Biochemistry 18*, 5770–5775.

Dudley, K. H., & Hemmerich, P. (1967) *J. Org. Chem. 32*, 3049–3054.

Gerig, J. F. (1968) *J. Am. Chem. Soc. 90*, 1065–1066.

Grande, H. J., Van Schagen, C. G., Jarbandhan, T., & Müller, F. (1977) *Helv. Chim. Acta 60*, 348–366.

Jensen, F. D., & Beck, B. H. (1968) *J. Am. Chem. Soc. 90*, 1066–1067.

Kierkegaard, P., Norrestam, R., Werner, P.-E., Csöregh, I., van Glehn, M., Karlsson, R., Leijonmark, M., Rönnquist, O., Stensland, B., Tillberg, O., & Torbjörnsson, L. (1971) in *Flavins and Flavoproteins* (Kamin, H., Ed.) pp 1–22, University Park Press, Baltimore, MD.

Lambert, J. B., & Oliver, L. W. (1968) *Tetrahedron Lett.*, 6187–6190.

Lambert, J. B., Oliver, L. W., & Packard, B. S. (1971) *J. Am. Chem. Soc. 93*, 933–937.

Lehn, J. M., & Wagner, J. (1970) *Tetrahedron 26*, 4227–4240.

Moonen, C. T. W., Vervoort, J., & Müller, F. (1984) *Biochemistry* (first paper of three in this issue).

Müller, F., & Massey, V. (1969) *J. Biol. Chem. 244*, 4007–4016.

Palmer, M. H., & Platenkamp, R. J. (1979) in *Catalysis in Chemistry and Biochemistry*, pp 147–169, Reider, Amsterdam.

Shanan-Atidi, H., & Bar-Eli, K. H. (1970) *J. Phys. Chem. 74*, 961–963.

Simondsen, R. P., & Tollin, G. (1980) *Mol. Cell. Biochem. 33*, 13–24.

Tauscher, L., Ghisla, S., & Hemmerich, P. (1973) *Helv. Chim. Acta 56*, 630–649.

Van Schagen, C. G., & Müller, F. (1980) *Helv. Chim. Acta 63*, 2187–2201.

Van Schagen, C. G., & Müller, R. (1981) *Eur. J. Biochem. 120*, 33–39.

Werner, P.-E., & Rönnquist, O. (1970) *Acta Chem. Scand. 24*, 997–1009.