First publ. in: Flavins and Flavoproteins, Proceedings of the 10th Internat. Symposium 1991, pp. 27-32

IONISATION PROPERTIES OF REDUCED, 1,5-DIHYDROFLAVIN, RATES OF N(5)-H EXCHANGE WITH SOLVENT

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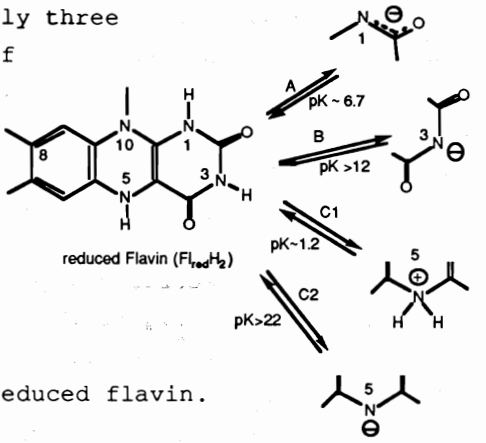
Introduction

Fully reduced flavin has primarily three functions at which ionisations of biochemical relevance occur:

- **A)** N(1)-H, pK 6.5-6.7
- **B)** N(3)-H, pK >12.
- **C)** N(5)-H which can
  - C1) protonate or
  - C2) deprotonate at low or high pH values.

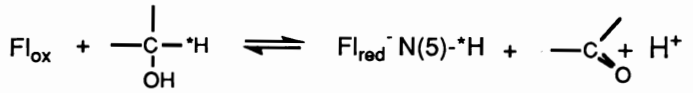


Scheme 1 Ionisations of fully reduced flavin.

A) The importance of the N(1)-H ionisation has long been recognized. It strongly influences the reactivity of the reduced molecule, in particular towards oxygen. A shift of this pK also reflects changes in the redox state of the isoalloxazine [1].
B) The ionisation of N(3)-H does not appear to have a major role in influencing the catalytic properties of the flavin. In some cases, e.g. with *oxidized* glycollate oxidase, the pK_a of N(3)-H is lowered from ~10 to ~6.7 due to the interaction with the

protein [2]. Whether a similar interaction occurs between the *reduced* flavin and the protein is not known. ^{15}N-NMR measurements at high pH (10-12) [3] give a pK_a >12 for the *reduced* flavin N(3)-H, and with all proteins studied so far, this function appears not to be ionized [4].

C) N(5)-H does not have a pK in or near the physiologically relevant range. However, the N(5) position is directly involved in the uptake and release of redox equivalents. Its electronic configuration undergoes the largest changes (sp^2 -> sp^3) during the transition between the oxidized and the reduced state, i.e. during catalysis. In particular, it can serve as the acceptor of the hydrogen bound to the substrate α-carbon:



Since N(5)-H has been found to exchange with solvent [5], the mechanism(s) of this exchange is of biochemical importance.

Dudley and coworkers [6] estimated the pK_a for the protonation of N(5)-H as <0 (C1 scheme 1), however, no experimental evidence was provided, and this value has been propagated unchallenged in the literature. We have measured a pK value of ~1.2 by monitoring the spectral changes of the reduced species as a function of pH (*cf* figure 1): Protonation occurs at position N(5) as deduced from the close similarity of the spectra of protonated reduced flavin and those of N(5)-dialkylated species reported earlier [7].

On the other hand, deprotonation of N(5)-H yields anionic N(5) (C2, scheme 1), which has been proposed to be a catalytic intermediate formed upon reaction of flavocytochrome b_2 with lactate [8]. This would confer a particular importance to this ionisation. The chemical shift of ^{15}N is correlated with the strength of an N-H bond i.e. to the pK_a value as is shown in figure 2. We have employed this correlation to estimate the pK_a

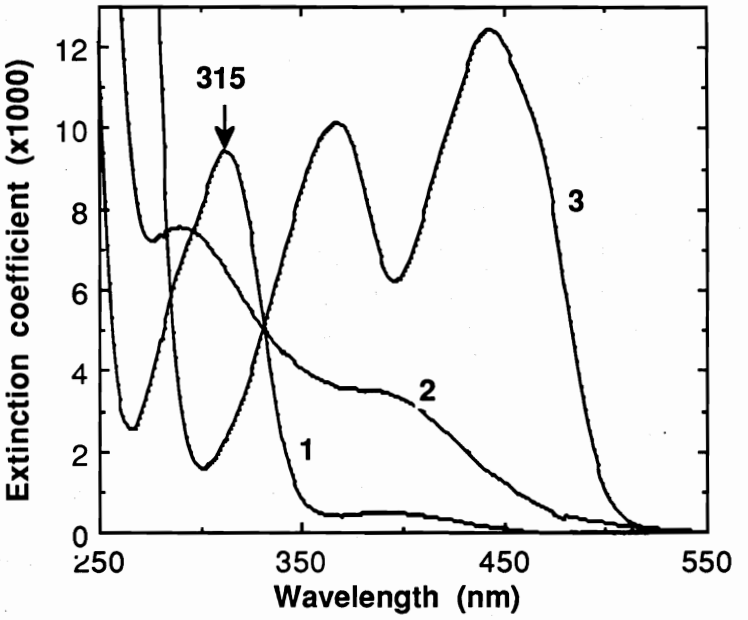


Figure 1: Absorption spectra of neutral and cationic reduced flavin, and of (neutral) oxidized flavin as comparison. An aqueous solution of 3-methyllumiflavin (~$3 \cdot 10^{-5}$ M) (curve **3**) was irradiated in the presence of EDTA (0.03M) at pH 5 to yield neutral reduced flavin, curve (**2**). To this, perchloric acid was added anaerobically to yield a 1 M solution. The resulting spectrum, (curve **1**), is corrected for dilution and for the presence of traces of cationic flavin radical. Whether the residual absorbance at ~ 400 nm belongs to the chromophore of cationic reduced flavin or to the presence of traces of protonated oxidized flavin cannot be determined.

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of N(5)-H using the chemical shift of ^{15}N(5) in reduced flavin. The pK_a of N(5) can also be estimated from the linewidth of the NMR-resonance. Reduced flavin N(5)-H has been shown to exchange with solvent in a number of cases, e.g. with flavocytochrome b_2 [5]. The rate of N(5)-H exchange with solvent has been assumed to be very high [10]. We have estimated it from the linewidth of

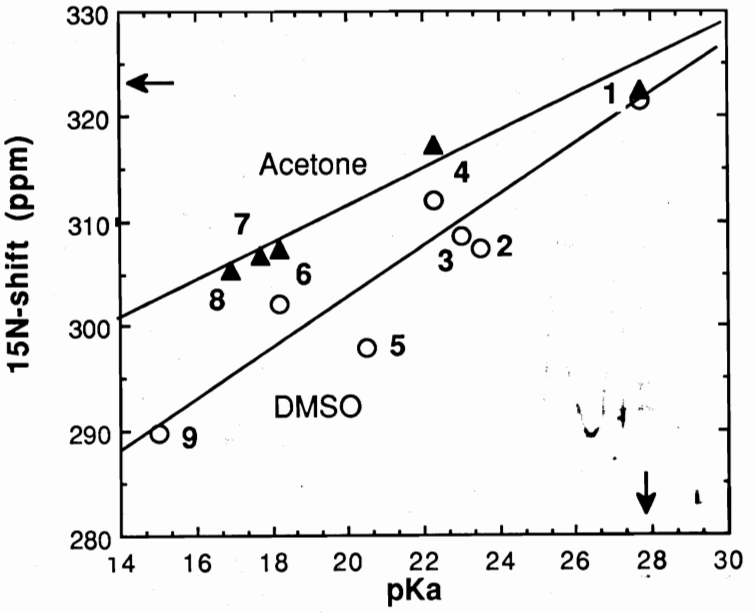

Figure 2: Estimation of the reduced flavin N(5) pK from the correlation of its ^{15}N-chemical shift with the pK_a of a series of aromatic amines. The values are from [3,9-11] (nitromethane as reference). The compounds are: **1**: aniline, **2**: 2-azaaniline; **3**: 4-cyano-; **4**: 4-aza-; **5**: 2,6-diaza-; **6**: 4-nitro-; **7**: 2-nitro-; **8**: 4-chloro-2-nitro-; and **9**: 2,3-dinitroaniline. Horizontal and vertical arrows: chemical shift of $^{15}N(5)$-H in reduced flavin [3,10,11] and value estimated for the N(5)-H pK_a.

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the $^{15}N(5)$ NMR resonance between pH 6 and 11. It reaches a minimum around pH 9.5, and, quite interestingly, the rate of exchange is not affected by the ionisation at N(1). This should be compared to the finding, that the ^{15}N-chemical shift of N(5) in reduced flavin also is not altered by deprotonation at N(1) [7-9]. Both findings are somewhat unexpected and will be discussed elsewhere [3]. From the rates of exchange the pK_b and pK_a values of N(5)-H can be estimated as ~-1.5 and ~22. The latter value is in reasonable agreement with the one derived from figure 2 in view of the difficulties inherent to the deter-

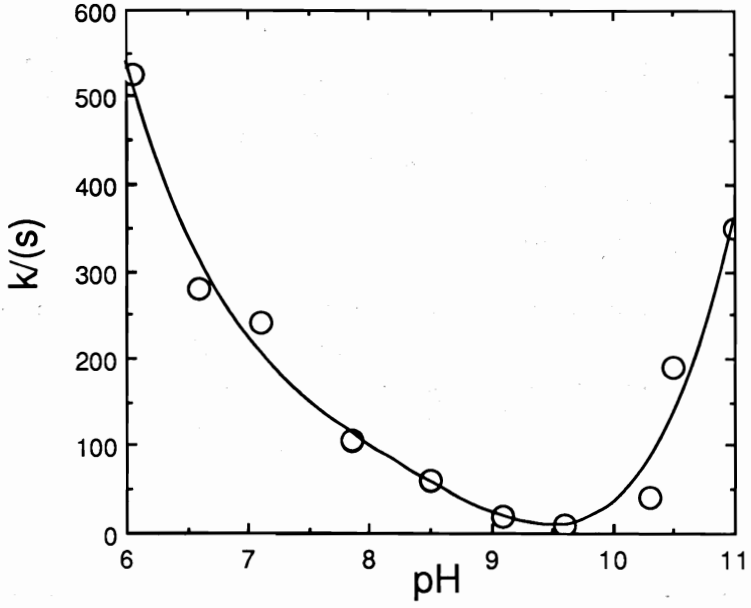

Figure 3:
pH dependence of the rate of N(5)-H exchange. The data were obtained from the line width of the ^{15}N-NMR signal of fully reduced flavin. Experimental details to be described elsewhere [3].

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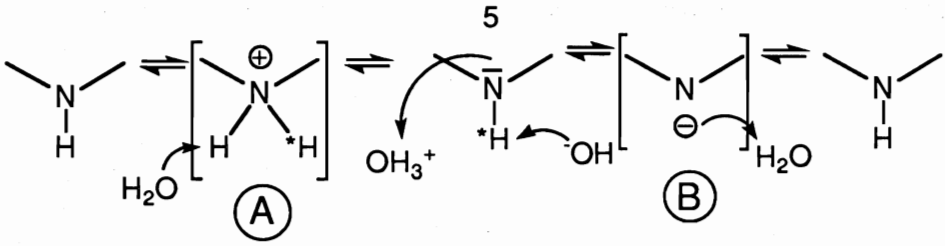
mination of the line width of ^{15}N-NMR signals. Our data suggest that N(5)-H does exchange by different mechanisms at high and low $[H^+]$. At low pH exchange might be initiated by protonation of N(5)-H by H_3O^+ *via* the cationic transient (**A**), (scheme 2), while at high pH the intermediate or transient species might be anionic (**B**) and would be formed by interaction with OH^-.



Scheme 2: Modes of exchange of N(5)-*H in fully reduced flavin with solvent. Left and right hand sides, modes of exchange at low and high pH.

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