THE KINETICS OF LUMIFLAVIN REDUCTION BY N-METHYL-1,4-DIHYDRONICOTINAMIDE: DIRECT EVIDENCE FOR A PREEQUILIBRIUM COMPLEX BETWEEN OXIDIZED LUMIFLAVIN AND N-METHYL-1,4-DIHYDRONICOTINAMIDE*

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The rates of anaerobic reduction of LF by NMNH exhibited saturation kinetics with a dissociation constant (K_1) of 0.11 M and a first order rate constant (k_2) of 7.1 sec^{-1}. Substitution of deuterium at the C-4 position of NMNH showed a 2.7 fold kinetic isotope effect on k_2 with no effect on K_1. During the reductive reaction, a long wavelength absorbing species was observed. The formation of this species was too fast to be measured by stopped flow techniques but its rate of disappearance was the same as the rate of reduction of the flavin. The turnover number of LF with O_2 and NMNH was significantly faster than the rate of anaerobic reduction of LF by NMNH. Therefore, the preequilibrium complex must react directly with O_2.

The existence of charge transfer complexes between reduced flavin and oxidized nicotinamide has been known for some time (1,2). However, evidence for complexes between reduced nicotinamide and oxidized flavin has been more elusive. Isenberg et.al. (3,4) first suggested that oxidized flavin and reduced nicotinamide form charge transfer complexes important in the oxidation of reduced nicotinamides by flavins. In subsequent kinetic studies of flavin reduction by N-propyl-1,4-dihydronicotinamide evidence for a complex between reduced nicotinamide and oxidized flavins was not found (5,6). Recently Bruice et.al. (7) have found a linear relationship between the binding of various isoalloxazines to tryptophan and their bimolecular rates of reduction

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Abbreviations used: LF-lumiflavin; LFH_2-1,5-dihydrolumiflavin; NMNH-N-methyl-1,4-dihydronicotinamide; NMN^+-N-methyl nicotinamide; v-initial velocity

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by N-propyl-1,4-dihydronicotinamide. Consequently, they suggested that a "kinetically important preequilibrium complex formation takes place between flavins and the dihydronicotinamide ring" in the course of isoalloxazine reduction.

In this paper we will present direct kinetic and spectrophotometric evidence for a complex between oxidized lumiflavin and reduced nicotinamide. We have chosen NMNH as the reduced nicotinamide since it is significantly more soluble in aqueous solvents than other dihydronicotinamides.

MATERIALS AND METHODS: LF was a gift of Richard Proffitt, Department of Biochemistry and Biophysics, University of California at Davis. Nicotinamide-1-methochloride was prepared by the method of Holman and Wiegand (8). NMNH and [4-^{2}H] NMNH were prepared as described by Suelter and Metzler (5) for N-propyl-1,4-dihydronicotinamide. Attempts at crystallization of the reduced product were unsuccessful. The product was dissolved in H_2O then extracted with $CHCl_3$ and dried *in vacuo*. This procedure was repeated twice. The resulting yellow oil (9) was stored at -10°C in the dark. The ^{1}H-NMR spectrum of NMNH in $CDCl_3$ (recorded on a Varian A-60 spectrophotometer) showed the same resonance patterns for the dihydronicotinamide ring protons as crystalline N-propyl-1,4-dihydronicotinamide in the same solvent. The ^{1}H-NMR spectrum of the deuterated product indicated 65% and 80% deuteration of the C-4 and C-2 positions of dihydronicotinamide respectively. NMNH solutions were prepared on the day of use and were calibrated with an extinction coefficient of 7.0×10^3 $M^{-1}cm^{-1}$ at 362 nm (10). Oxygen measurements were made on a Clark electrode. Spectrophotometric measurements were made either on a Cary 14 or a Durrum-Gibson stopped flow spectrophotometer. All reactions were measured at 25°C in 0.5 M KCl, 0.01 M Tris-HCl at pH 8.0.

RESULTS AND DISCUSSION: Anaerobic reduction of LF by NMNH was monitored on the stopped flow spectrophotometer at 470 nm. First order plots of the absorbance decrease gave straight lines for at least 90% of the reaction. The upper

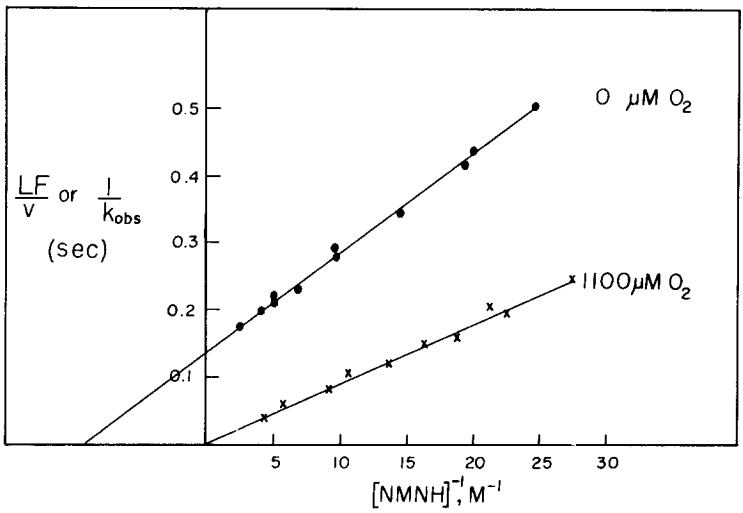

Figure 1. The upper trace (-O-) is a plot of the inverse of the anaerobic rate of reduction of LF measured at 470 nm versus $[NMNH]^{-1}$. After mixing in the stopped flow spectrophotometer the LF concentration was 42 μM. The lower trace (-x-) is a plot of $(v/LF)^{-1}$ measured on the O_2 electrode in the presence of 1.1 mM O_2 versus $[NMNH]^{-1}$. The flavin concentration was 1.35 μM. Other reaction conditions are given in the methods section.

line of Figure 1 is a plot of the reciprocal of k_{obs} for LF reduction versus the reciprocal of NMNH concentration. The rate of anaerobic reduction of LF by NMNH saturates at 7.1 sec^{-1} (k_2) with a dissociation constant of 0.11 M (K_1). Using deuterated NMNH a 1.75 fold kinetic isotope effect is found on k_2 (k_2^{1H}/k_2^{2H}) and no effect is observed on K_1. Correcting for complete deuterium substitution at the C-4 position of dihydronicotinamide an isotope effect of 2.7 is calculated. Thus, it may be concluded that a kinetically importantly complex between the reduced nicotinamide and oxidized flavin occurs prior to the reduction of the flavin.

If one measures O_2 consumption by NMNH in the presence of LF and expresses the observed initial velocity as a first order rate constant (i.e. v/LF, sec^{-1}), the resulting double reciprocal plot at 1.1 mM O_2 is given by the bottom line of Figure 1. It is seen that the rate of O_2 consumption is significantly faster than the anaerobic rate of reduction of the flavin. If the overall reaction proceeded only through reduced flavin, the turnover number of the flavin would be less than the rate of anaerobic reduction of the flavin. Thus the

complex formed prior to the reduction of LF must be able to react directly with O_2. After correction for complete deuterium substitution at the C-4 position a 2.5 fold isotope effect was found on the slopes of the double reciprocal turnover plot. Eventhough an intercept at infinite NMNH in the turnover experiments is not experimentally measurable, the deuterium isotope effect suggests that carbon hydrogen bond cleavage is partially rate determining when O_2 reacts with the preequilibrium complex to give products.

During the anaerobic reduction of LF by NMNH the disappearance of a transitory intermediate absorbing at long wavelength was observed on the stopped flow spectrophotometer. A plot of the total change of 550 nm absorbance versus NMNH concentration resulted in a saturation curve. The dissociation constant was similar to that found in Figure 1 for the kinetics of LF reduction suggesting that this intermediate is probably the preequilibrium species responsible for the saturation kinetics of LF reduction. A stopped flow spectrum of the intermediate complex is given in Figure 2. The extinction coefficients have been calculated from the total absorbance change after anaerobic mixing of LF and NMNH in the stopped flow and have been corrected for complete saturation of the reaction. The spectrum was not measured at wavelengths below 460 nm because of the increasing absorbance of NMNH. At long wavelengths where only the complex absorbs, formation of the complex at an NMNH concentration equal to K_1 was too fast to be measured by the stopped flow method and justifies calling K_1 a true dissociation constant. The rate of disappearance of long wavelength absorbance was identical to the rate of formation of LFH_2.

It should be emphasized that the long wavelength absorbing species observed here can not be attributed to either the semiquinone of LF or a charge transfer complex between LF and LFH_2 (11), since the concentration of the intermediate is maximal when the concentration of oxidized flavin is maximal. In addition this species cannot be attributed to a complex between reduced flavin and oxidized nicotinamide for two reasons: the complex occurs prior to cleavage of the C-4 carbon hydrogen bond of the dihydronicotinamide as shown by the

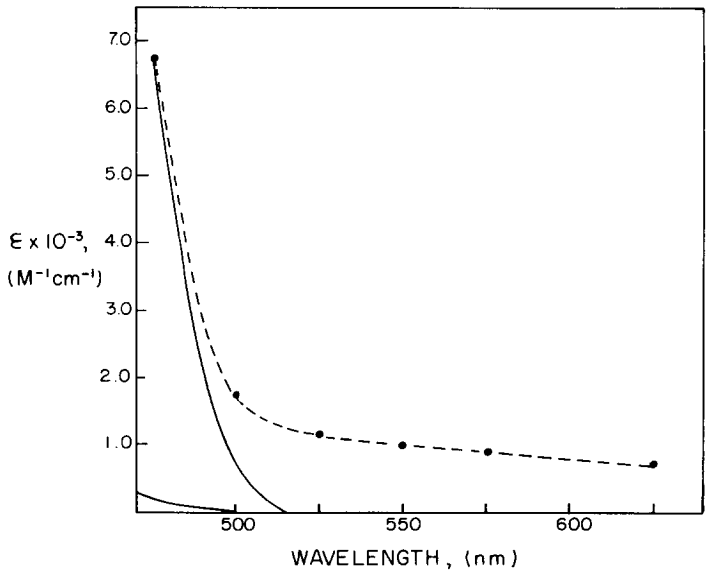

Figure 2. The upper and lower solid lines represent LF and LFH_2 respectively. The dashed line is the spectrum of the intermediate measured on the stopped flow. After mixing the LF concentration was 42 μM and the NMNH concentration was 0.38 M. The spectrum shown is corrected for incomplete saturation (factor of 1.3). Corrections were also made for the effect of mixing NMNH at this concentration with buffer. Other conditions are given in methods section.

deuterium isotope effect and the spectrum of the intermediate has oxidized flavin character in contrast to the reported spectra of reduced flavin-oxidized nicotinamide charge transfer complexes (2). The intermediate we observe must be a complex between oxidized flavin and reduced nicotinamide.

Our results may be summarized as follows:

$$\begin{array}{ccccccc} LF + NMNH & \overset{K_1}{\rightleftharpoons} & LF\cdots NMNH & \xrightarrow{k_2} & LFH_2 + NMN^+ \\ & & \Big\downarrow k_3 \; O_2 & & \Big\downarrow k_4 \; O_2 \\ & & H_2O_2 + LF + NMN^+ & & LF + H_2O_2 \end{array} \qquad (1)$$

The experimentally observed long wavelength absorbing intermediate is attributed to the complex LF···NMNH formed in the rapid preequilibrium step defined by K_1. We have demonstrated that the NMNH in this complex is in an activated state capable of reacting with O_2 by the bimolecular step k_3. Thus under

aerobic conditions the NMNH in the complex may donate reducing equivalents directly to molecular O_2 while under anaerobic conditions the complexed NMNH donates reducing equivalents by the step k_2 to the flavin moiety. In both cases carbon hydrogen bond cleavage is partially rate limiting.

Finally, the extreme similarity of the optical spectrum of our intermediate to those reported by Massey et.al. (12) in the enzyme catalyzed flavin dependent dihydronicotinamide dehydrogenation should be noted. To our knowledge it is the first time that the spectrum of such an intermediate has been demonstrated in a model system. Based on the results shown in this paper we propose that the intermediates reported by Massey et.al. (12) represent complexes (probably of the charge transfer type) between protein bound oxidized flavin and dihydronicotinamide.

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References

1. V. Massey and G. Palmer, J.Biol.Chem. 237, 2347 (1962).
2. T. Sakurai and H. Hosoya, Biochim.Biophys.Acta. 112, 459 (1966).
3. I. Isenberg and A. Szent-Györgyi, Proc. Nat. Acad. Sci. U.S. 45, 1229 (1959).
4. I. Isenberg, S.L. Baird and A. Szent-Györgyi, Proc. Nat. Acad. Sci. U.S. 47, 245 (1961).
5. C.H.Suelter and D.E. Metzler, Biochim. Biophys. Acta. 44, 23 (1960).
6. G.K. Radda and M. Calvin, Biochemistry. 3, 384 (1964).
7. T.C. Bruice, L. Main, S. Smith, and P.Y. Bruice, J. Amer. Chem. Soc. 93, 7327 (1971).
8. W.I.M. Holman and C. Wiegand, Biochem. J. 43, 423 (1948).
9. D. Mauzerall and F.H. Westheimer, J. Amer. Chem. Soc. 77, 2261 (1955).
10. G. W. Rafter and S.P. Colowick, J. Biol. Chem. 209, 773 (1954).
11. J.L. Fox and G. Tollin, Biochemistry. 5, 3865 (1966).
12. V. Massey, R.G. Matthews, G.P. Foust, L.G.Howell, C.H. Williams, G. Zannetti and S. Ronchi in Pyridine Nucleotide Dependent Dehydrogenases, ed. H. Sund, Springer Verlag Berlin, 393-411. (1970).