Kinetics and Isotope Effects for Transhydrogenation from a NADH Model Compound to Flavins

by

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Kinetics and isotope effects for the reaction of a Hantzsch ester and a monodeuteriated Hantzsch ester with five isoalloxazines have been studied. The kinetic deuterium isotope effect (k_H/k_D) for "usual" isoalloxazines is in good accord with the paritioning isotope effect (Y_H/Y_D) and the secondary isotope effect (k_H/k'_H) is almost equal to unity. On the other hand, k_H/k_D for electron-deficient isoalloxazines is greater than Y_H/Y_D, and k_H/k'_H significantly exceeds unity. The results suggest that one-step hydride-like transfer occurs in "usual" isoalloxazines, whereas hydrogen transfer to electron-deficient isoalloxazines may proceed according to the multi-step transfer mechnism. We thus consider that the hydrogen transfer mechanism changes depending on the electron-dificiency of inoalloxazines, although the possibility that the isotope scrambling is responsible for the discrepancy is not excluded completely.

It is known that reductions by NADH have invariably occur by direct transfer of a proton plus two electrons to the substrate molecule, and one may envisage the mechanism of hydrogen transfer fairly convincingly[2)]. This is due to the fact that a hydrogen from NADH is "fixed" on a carbon atom of the reduced products. On the other hand, the mechanism of the biochemically important oxido-reduction between NADH and flavin is hardly understood. The difficulty stems from the fact that the protons of the ultimate product (1, 5-dihydroflavin) are bound to weakly basic nitrogens and are, therefore, exchangeable. It was proposed in former days that the hydrogen transfer might proceed via a covalent intermediate between NADH and flavin[3)], but a number of recent studies on the NADH model reduction suggest that the hypothesis is highly unlikely[2,4-12)]. Thus, two possible mechanisms remain : (a) concerted, one-step transfer of a hydride ion and (b) multi-step transfer of $e+H^++e$ (or $e+H\cdot$) via a radical ion-pair intermediate.

In 1961, Drysdale et al.[13)] demonstrated that the hydrogen transfer from NADH to flavin (or from reduced flavin to NAD^+) occurs by direct hydrogen transfer in cytochrome b_5 reductase. On should note, however, that such enzymatic events in shielded active sites frequently lack the equilibrium with solvent hydrogen. As an alternative method, 5-deazaflavin in which 5-nitrogen of flavin is substituted by a carbon was used as a hydrogen acceptor and evidence for direct hydrogen transfer was offered not only in the model system but also in the enzymatic system[14),15)]. Hemmerich and Jorns[16)] commented, however, that 5-deazaflavin might be a NAD^+- rather than a flavin-model. Meanwhile, Blankenhorn[17)] proposed that the slopes of the linear free energy relationships are indicators of whether the transfer of the hydrogen nucleus is accompanied by one or

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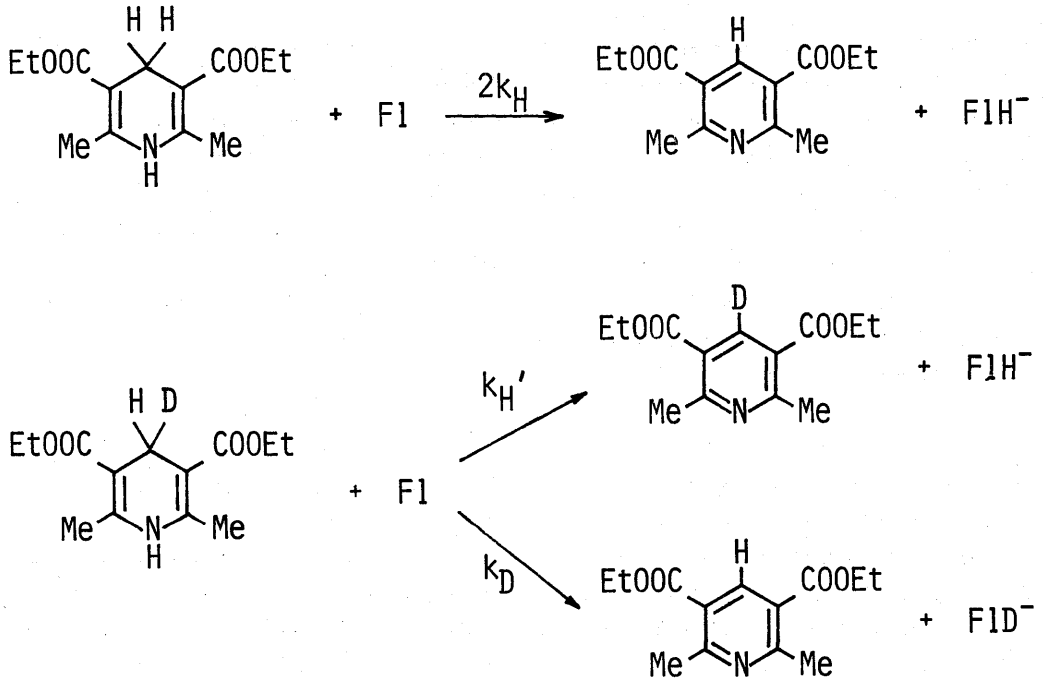
by two electrons, but Kurz and Kurz[18] later argued against this proposal. It seems to us, therefore, that studies on the mechanism of hydrogen transfer from HADH to flavin so far have suffered from the problem that no firm experimental tools are available to distinguish between two major possible mechanisms.

In the NADH model reduction of carbonyl and acridinium substrates, the mechanism has been discussed on the basis of comparison of the kinetic deuterium isotope effect (k_H/k_D) with the product H/D isotopic ratio (Y_H/Y_D). In one-step reactions, k_H/k_D must be in accord with Y_H/Y_D, whereas the discrepancy between these two values suggests the presence of at least one intermediate along the reaction process[2,4-12]. It occurred to us that the product H/D radio in the reaction of NADH and flavin can be determined by analyzing the content of deuterium remaining in the product NAD^+. With this idea in mind, we carried out the reaction of a Hantzsch ester (2, 6-dimethyl-3, 5-dicarboethoxy-1, 4-dihydropyridine : HH) and a mono-deuteriated Hantzsch ester (HD)[19,20] with five isoalloxazines (Fl)[21].

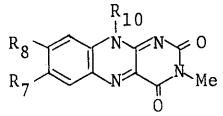


Scheme 1

The kinetic measurements were carried out at 30°C in aerobic 10 vol% aqueous ethanol (pH 8.12 with 0.01 M borate buffer) unless otherwise stated and the progress of the reaction was followed spectrophotometrically by monitoring the decrease in the absorption band of HH (or HD) at 372 nm. In the product analysis, 2, 6-dimethyl-3, 5-dicarboethoxypyridine in the preparative-scale reaction mixture was isolated by tlc[22] and the content of deuterium was deternied by mass spectroscopy, The results are summarized in Table 1.

In Table 1, several points may be worthy to be mentioned. First, both k_H/k_D and Y_H/Y_D are relatively small as a primary isotope effect and are in good accord with the kinetic isotope effect previously determined for the reaction of 1-propy-1, 4-dihydronicotinamide and riboflavin ($k_H/k_D=3.2$)[23]. The magnitude of the isotope effects implies that the hydrogen transfer would be involved only partially in the rate-determining step. Secondly, the kinetic isotope effects for the isoalloxazines without electron-withdrawing substituent(s) show a satisfactory agreement with the partitioning isotope effects. Furthermore, the secondary isotope

Table 1 Kinetic and Product Isotope Effects in the Reaction of Isoalloxazines with HH (Hantzch ester) and HD (monodeuteriated Hantzsch ester)[a]



R_7	R_8	R_{10}	k_r[c]	k_r^D[d]	k_r/k_r^D	k_H/k_H'[e]	k_H/k_D[f]	Y_H/Y_D[g]	$k_H/k_D - Y_H/Y_D$
			(M^{-1} s^{-1})						
H	H	Et	47.1	32.1	1.47	0.98	2.90	2.95	-0.05
H	H	Ph[b]	90.1	56.3	1.60	1.09	3.01	2.75	0.26
H	Cl	Et	100	61.4	1.63	1.16	2.74	2.39	0.35
Cl	Cl	Me	370	219	1.69	1.17	3.04	2.64	0.40
CN	H	Me	548	272	2.01	1.39	3.63	2.63	1.00

[a] 30°C, 10 vol% ethanol, pH 8.1 with 0.01 M borate. In the kinetic measurement: [HH or HD] = 6.70×10^{-5} M, [isoalloxazine] = 5.00×10^{-5} M. In the product analysis: [HH or HD] = [isoalloxazine] = 2.95×10^{-4} M. We confirmed that the same rate constants and Y_H/Y_D are obtained under following concentration range: [isoalloxazine] = (5-32) $\times$ 10^{-5}M for the kinetics and [HH or HD] = [isoalloxazine] = (0.1-1.2) $\times 10^{-3}$ M for the analysis

[b] 20.6 vol% ethanol.

[c] Second-order rate constants for the reaction of HH with isoalloxazines.

[d] Second-order rate constants for the reaction of HD with isoalloxazines. The data are corrected for the deuterium purity.

[e] $$\frac{k_H}{k_H'} = \frac{1}{2}\left(\frac{k_r}{k_r^D}\right)\left(1 + \frac{Y_D}{Y_H}\right)$$

[f] $$\frac{k_H}{k_D} = \frac{(k_r/k_r^D)}{2-(k_r/k_r^D)(k_H'/k_H)}$$

[g] Product isotope partitioning was estimated from the amount of hydrogen (or deuterium) remaining on 2,6-dimethyl-3,5-dicarboethoxypyridine. The data are corrected for the deuterium purity.

effects (k_H/k_H') are almost equal to unity as generally expected for the reactions involving the conversion of sp^3-carbon to sp^2-carbon.[24]. Hence, the oxidation reduction between Hantzsch esters and these isoalloxazines can be regrarded as an apparent one-step reaction. Thirdly and most importantly, a clear disparity between k_H/k_D and Y_H/Y_D exists in the isoalloxazines with electron-with-drawing substituent(s). Apparently, k_r/k_r^D increases with increasing electron-deficiency of isoalloxazines while Y_H/Y_D is almost constant, and in particular, 3, 10-dimethyl-7-cyanoisoalloxazine gives $k_H/k_D - Y_H/Y_D = 1.00$ and $k_H/k_H' - 1.0 = 0.39$. The disparity indicates that one may propose the existence of at least one intermediate along the reaction pathway for the reaction with the electron-deficient isoalloxazines. If such an intermediate really exists, the most probable intermediate would be the charge-transfer complex or the radical ion-pair complex[25-26]. Fanally, the disparity reported in the past literatures features $k_H/k_D < Y_H/Y_D$ and $k_H/k_H' < 1.0$.[2,4-12] In contrast, the data for the electron-deficient isoalloxazines in Table 1 reveal that k_H/k_D is greater than Y_H/Y_D and k_H/k_D is greater than unity. We cannot explain the origin of this anomaly clearly.

Recently, Powell and Bruice[27] reported that the discrepancy between k_H/k_D and Y_H/Y_D can be accomodated by isotope scrambling. Since the attention to this problem in not paid, the results of the present paper must be partly discounted. At present, we consider that if hydrogen transfer from Hantzsch ester to

3, 10-dimethyl-7-cyanoisoalloxazine really proceeds via a multi-step mechanism, the intermediate must be profoundly stabilized by 7-cynao group.

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