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ON THE ORIGIN OF HETEROGENEITY OF FLUORESCENCE DECAY KINETICS OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE

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SUMMARY: The fluorescence lifetimes of reduced nicotinamide adenine dinucleotide and other dihydronicotinamide derivatives were measured by picosecond laser excited time correlated single photon counting technique. All the dihydronicotinamide derivatives (including the simple model compound N-methylnicotinamide) had fluorescence decay profiles which could be fitted to double and triple exponentials in neutral aqueous solutions and in dimethyl sulfoxide respectively. It was concluded that the heterogeneity in the measured lifetimes arises from the inherent photoprocess of the dihydronicotinamide chromophore and not due to any intramolecular interaction as assumed in earlier studies. Some of the possible schemes for the fluorescence decay are discussed.

Nicotinamide adenine dinucleotide, NAD^+ and its reduced form, NADH, play a major role in biological oxidation-reduction reactions. There are more than 300 enzymes which use NAD^+/NADH as coenzyme. During our work in characterizing the nature of binding of NADH to mitochondrial NADH-coenzyme quinone oxidoreductase and other dehydrogenases using its fluorescence, we noted that the fluorescence of NADH itself has not been characterized well. In the past, both steady state (1-3) and time-resolved (4-6) fluorescence of NADH and NADPH have been used to study the binding to enzymes. The biexponential fluorescence decay of enzyme-bound nucleotide observed in time-resolved studies has been explained as due to either a reversible excited state reaction (5) or presence of two binding sites for the nucleotide (6). However, recent studies (7,8) using mode-locked lasers have shown that the fluorescence decay of NADH and its analogs in aqueous solutions are multiexponential even in free form. Heterogeneity in the ground state or in the excited state were invoked in explaining the multiexponential decay in the free form (7,8). The

Abbreviations: NADH, β-nicotinamide adenine dinucleotide, reduced form; NMNH, β-nicotinamide adenine mononucleotide, reduced form; MNH, 1-methylnicotinamide, reduced form; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; AADH, 3-acetylpyridine adenine dinucleotide, reduced form; HFL, 3-hydroxyflavone; DMSO, dimethylsulfoxide; HPLC; high performance liquid chromatography; NMR, nuclear magnetic resonance.

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'open' and 'folded' conformations of NADH inferred from NMR studies (9-11) were attractive candidates in describing the ground state heterogeneity (7).

In this communication, we address the question on the origin of multi-exponential fluorescence decay of NADH in the free form. The answer to this question is essential for using the fluorescence of NADH to infer the nature of binding to enzymes. Our results show that the origin lies in the intrinsic photophysics of the dihydronicotinamide chromophore.

MATERIALS AND METHODS

Chemicals and purifications: NADH, α-NADH, NMNH, NADPH and AADH were obtained from Sigma Chemical Co. MNH was synthesised as reported (12). These nucleotides and analogs were purified using a μBondapak C_{18} reverse phase column (Waters) and LKB - HPLC system (13). 0.1 M KH_2PO_4, pH 7.15 containing 5% methanol was used as the mobile phase. The main peak was collected, lyophilised and dissolved either in 10 mM KH_2PO_4, pH 7.0 or in DMSO. These samples were immediately taken for fluorescence lifetime measurements. The concentration of these samples were in the range 10 - 100 μM.

Measurement of fluorescence lifetimes: The fluorescence lifetimes were measured by the technique of picosecond laser excited time correlated single photon counting (14). The details of the experimental set up will be published elsewhere. The excitation UV (310 nm) laser pulses (800 kHz) were vertically polarized. Fluorescence emission polarized at 54.7° was detected at a count rate < 8000 per second. The fluorescence decay data were collected with a peak count of $2x10^4$ - $5x10^4$ at 42 picoseconds/channel. Initially we used the conventional data analysis using the instrument response function (IRF) obtained at 310 nm using a scattering medium and fluorescence emission at 450 nm for NADH. The two lifetimes (0.18 and 0.66 ns) for NADH were in agreement with reported values (7), but the goodness of fit criteria were not always acceptable. Significant improvement in the goodness of fit criteria was observed, without change of lifetimes, by adopting the excitation pulse-shape mimic technique (15). This latter technique overcomes certain instrument anomalies associated with large difference in excitation and emission wavelength. Fluorescence lifetime as low as 10 ps has been unambiguously measured (16). The results reported in this communication were obtained using HFL in methanol as the pulse shape mimic compound. The fluorescence lifetime of HFL at 420 nm is measured to be 30±10 picoseconds, in agreement with the earlier report (17). Deconvolution analysis was done by iterative reconvolution method with parameter adjustment by Marquart procedure in successive iterations. The pulse shape mimic technique using HFL and deconvolution analysis gave excellent fit for the fluorescence decay of a standard sample, 2,2' p-phenylene-bis-(5-phenyloxazole) (POPOP) in methanol: lifetime = 1.32 ns chi-square = 1.25, DWP = 1.64, ZRUN = -0.9 and PER = 94%.

RESULTS AND DISCUSSION

Fluorescence decay in neutral aqueous solutions: The fluorescence decay of NADH in neutral aqueous solution was clearly biexponential (Table I) in agreement with a previous report (7). The decay parameters did not show any significant variation in the pH range 4-9 and the emission wavelength in the range 400-500 nm (data not shown). Visser and van Hoek (7) assigned the shorter and the longer lifetimes to the 'open' and 'folded' forms of NADH, respectively. Energy transfer (18) and NMR (11) studies had shown that the relative concentration of the 'folded' form gets reduced by 50% in 50% methanol. We found that the decay parameters (especially the preexponential factors) did not show any appreciable change in 50% methanol or in 3M urea which is expected to

TABLE I. Fluorescence decay parameters[a] of NADH and related compounds in various solvents. The buffer used was 100 mM phosphate, pH 7.0 in all the cases. DMSO solutions had 100 mM phosphate

Sr. No.	Compound	Solvent	Emission wavelength nm	Temp. °C	A_1	τ_1 ns	A_2	τ_2 ns	A_3	τ_3 ns
1.	NADH	Buffer	450	25	10.0	0.21	3.9	0.53	-	-
				20	(10.0	0.25	2.2	0.69)[b]	-	-
				4	10.0	0.31	3.1	0.80	-	-
2.	NADH	50% Methanol-50% Buffer	450	25	10.0	0.25	3.4	0.66	-	-
3.	NADH	3M Urea in Buffer	450	25	10.0	0.20	3.6	0.55	-	-
4.	NMNH	Buffer	450	25	10.0	0.17	1.1	0.46	-	-
				5	10.0	0.21	1.5	0.55	-	-
5.	MNH	Buffer	450	25	10.0	0.18	1.9	0.46	-	-
6.	NADPH	Buffer	450	25	10.0	0.18	3.2	0.51	-	-
7.	α-NADH	Buffer	450	25	10.0	0.29	4.6	0.98	-	-
8.	AADH	Buffer	470	25	10.0	0.095	0.41	0.31	-	-
9.	NADH	DMSO	450	25	10.0	0.18	5.8	1.18	0.52	2.9
				5	10.0	0.17	6.7	1.38	2.60	2.9
10.	NMNH	DMSO	450	25	10.0	0.17	4.7	1.12	0.21	3.2
				5	10.0	0.17	4.2	1.28	0.39	3.8
11.	MNH	DMSO	450	25	10.0	0.17	13.2	1.04	0.50	3.1
12.	NADPH	DMSO	450	25	10.0	0.20	6.6	1.19	3.0	3.3
13.	α-NADH	DMSO	450	25	10.0	0.25	13.9	1.19	3.7	2.2
14.	AADH	DMSO	450	25	10.0	0.22	1.7	0.83	0.24	4.4
15.	NADH	DMSO	420	25	10.0	0.20	8.2	0.88	0.19	2.6
16.	NADH	DMSO	490	25	10.0	0.20	8.9	0.82	1.48	2.4
17.	NADH	Acetonitrile	450	25	10.0	0.23	10.6	1.41	8.76	3.5
18.	NADH	Methanol	450	25	10.0	0.20	7.2	0.63	0.15	2.0

[a] The values of Chi-square for these data were in the range 1.0-1.3. The uncertainties in the parameters τ_1, τ_2 and τ_3 were about 20%, 10% and 30% respectively. Two exponential fits in nonaqueous solvents gave Chi-square values greater than 5.0 for all compounds.

[b] Data from ref. 7.

break any H-bonding thereby preventing formation of 'folded' form. Hence, it is concluded that the folding of the dinucleotide is not the cause of heterogeneity in fluorescence lifetimes.

In order to support the above conclusion we measured the fluorescence decay kinetics of NMNH which lacks the adenine moity and MNH which lacks even the sugar ring. Fig.1A shows that a single exponential fit is clearly inadequate in the case of NMNH in aqueous solution. A biexponential fit was necessary and sufficient as seen from the distribution of weighted residuals and autocorrelation. Similar observations were made in the case of MNH also. The necessity of biexponential fit in these cases is in contrast to the observations of Visser and van Hoek (7). The observed biexponential decay in the cases of NMNH and MNH is not likely to be due to any fluorescent impurity, since our samples were purified by reverse phase - HPLC. The relative proport-

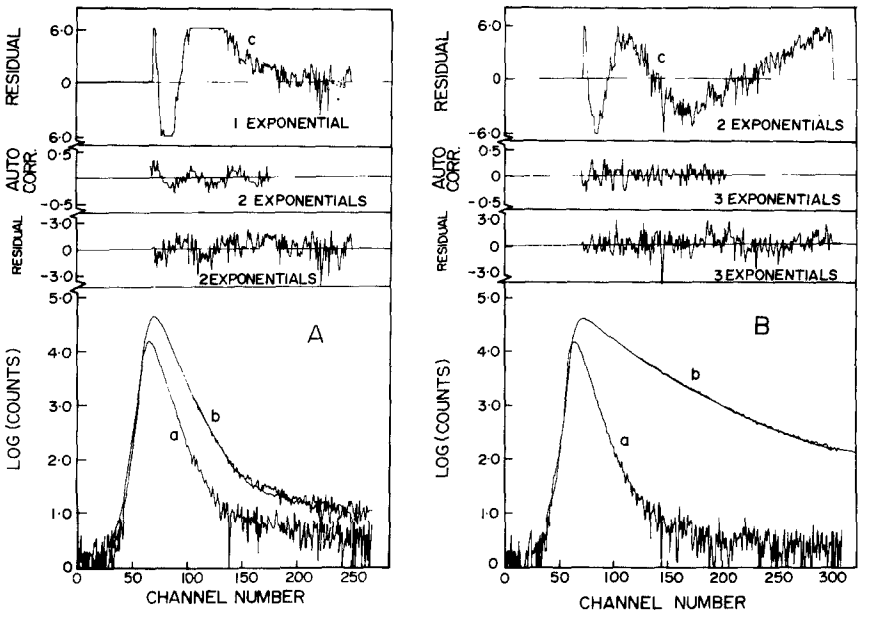

Fig. 1A. Typical fluorescence decay profile (trace b) of NMNH in 100 mM phosphate, pH 7.0 at 25°C. The 310 nm excitation UV pulse was generated by frequency doubling 620 nm dye laser pulse (pulse width <10 ps) which in turn was generated in a cavity-dumped synchronously-pumped Rhodamine 6G dye laser pumped by the frequency doubled output of a CW-mode-locked Nd/YAG laser (Spectraphysics). The fluorescence was monitored at 450 nm. For details of measurements see 'Materials and Methods'. Trace a is the fluorescence decay profile of HFL at 420 nm used in deconvoluting trace b (see Methods). The smoothline in trace b was computed for two exponentials with parameters given in Table I, line 4 and Chi-square 1.3. Single-exponential fit gave the lifetime as 0.24 ns with Chi-square 19.0 and the residuals were correlated (trace C). Peak counts of traces a and b are 20,000 and 43,000 respectively.

Fig. 1B. Analysis of the decay profile of NADH in DMSO (trace b) for two and three exponential fits. Other experimental conditions were the same as in Fig. 1A. The smoothline in trace b was calculated for three exponentials with parameters listed in Table I, line 9 and Chi-square 1.1. Two exponential fit gave A_1 = 10.0, τ_1 = 0.43 ns, A_2 = 5.4, τ_2 = 1.64 ns with chisquare = 11.6 and the residuals were correlated (trace c). Peak counts of traces a and b are 21,000 and 40,000 respectively. The time per channel was 42 ps for both A and B.

ion of the decay component with the longer lifetime was found to be about 3 times smaller in the case of NMNH when compared to NADH. This may be one of the reasons for the assignment of single exponential fit by Visser and van Hoek (7) in the case of NMNH. It may be pointed out that their fit of NMNH decay to a single exponential was worse than their fit of NADH decay to double exponential (The value of their goodness of fit parameter, which includes instrumental systematic errors also, was nearly twice in the case of NMNH when compared to that of NADH). The 30 ps decay component observed in NADH and in

an analog by streak camera measurements (8) at 380 nm (with excitation at 355 nm) was not observed in our experiments with excitation at 310 nm.

Fluorescence decay in DMSO: In neutral aqueous solutions NADH and its analogs, with the exception of AADH (which is not a nicotinamide derivative), have very similar values for the two lifetimes (Table I). This shows that the two lifetimes are inherent to the dihydronicotinamide moiety. However, since the relative proportion of the component with the longer lifetime is larger in dinucleotides when compared to mononucleotides (Table I), one could presume some intramolecular interactions (like folding) contributing to that component. In order to have further support to our thesis that the heterogeneity in the lifetime is not due to any intramolecular interaction, we measured the decay profiles in DMSO which is expected to breakup any intramolecular interaction. As seen from the analysis (Fig. 1B and Table I), NADH and analogs showed triple exponential decay in DMSO. Although the heterogeneity in fluorescence lifetimes showed an increase on going from aqueous solutions (double exponential) to DMSO (triple exponential) the results were more unified in DMSO. The decay parameters were very similar for NADH and the mononucleotide NMNH supporting our thesis that the heterogeneity in the lifetime is intrinsic to the chromophore.

Fluorescence decay schemes: Three exponential fits to decay profiles observed in DMSO were not special to this solvent alone. We found that triple exponential fits were necessary and sufficient to characterize the decay profiles in solvents such as acetonitrile and methanol (Table I). Double exponential fits were clearly inadequate in these cases as judged by the fit parameters (data not shown). Decay profiles in glycerol were also fitted by three exponentials (5). The observed double exponential fits to decay profiles in aqueous solutions might have been the result of reduced proportion of the third compoment. Further, it was found that the data could be fitted to two exponentials in aqueous solutions and to three exponentials in nonaqueous solvents with excellent goodness of fit parameters for all the compounds in the temperature range 5 - 25°C. Table I gives representative data for NADH and NMNH at 5°C and 25°C. However, we cannot rule out a more complex scheme involving multi-exponentials (more than three). This is generally true in any kinetic study where the experimental data is adequately fitted for a decay function with fewer free-parameters. Relying on our triple exponential fits one could say that any proposed scheme should account for the three time constants associated with the photoprocess of the dihydronicotinamide alone. The decay parameters did not vary significantly in the concentration range 0.06 - 4.0 mM in DMSO (data not shown) ruling out any contribution of intermolecular interaction. However, we observed a dependence on the emission wavelength. The normalised preexponential factor A_3 increased with increase in the emission wavelength in

DMSO (Table I, lines 9, 15 and 16). A_1 and A_2 did not show any significant variation. This indicates the presence of more than one emitting species at least in DMSO.

In conclusion, our results show that fluorescence decay kinetics of NADH is controlled by the intrinsic photoprocess of the chromophore, dihydronicotinamide, and not by any intramolecular interaction such as folding and stacking. Assignment of a specific decay scheme would require further experimentation in aqueous and other solvents.

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