

NADH in Solution: A Single State

Joachim Hönes^{§§}, Julia Wack^{**}, Katja Schmitz^{**}

^{§§}: to whom correspondence should be addressed: joachim.hoenes@gmail.com

^{§§}: Rodauer Straße 50a, 64673 Zwingenberg, Germany

^{**}: Technische Universität Darmstadt, Fachgebiet Biologische Chemie, Alarich-Weiss-Straße 8, 64287 Darmstadt, Germany

Summary

Gregorio Weber observed FRET from adenine to dihydronicotinamide (DHN) in NADH when the former was excited at 260 nm. He concluded that an intimate contact of bases was necessary. Spectroscopic and NMR studies have been performed leading to a conformation model with base stacking and DHN ring puckering and an equilibrium of folded and unfolded states. An exciplex of adenine with excited DHN in the folded form was assumed in order to explain the two fluorescence lifetimes. Many observations were interpreted using the two-state folded/unfolded model but quantitative agreement on the equilibrium position could not be achieved. The claim that all dinucleotides irrespective of oxidation and excitation state would exist with significant shares of both folded and unfolded forms i.e. with similar equilibrium constants near 1:1 is certainly surprising.

Counterarguments not discussed before can be derived from published data:

- Experiments with a model compound demonstrated efficient FRET at a distance of ~0.5 nm between adenine and DHN under denaturing conditions. Thus intimate contact is not a prerequisite for FRET.
- Sharp proton resonances and gradual drifts with environment changes led to the conclusion of folded/unfolded interconversion faster than the NMR timescale of nanoseconds. This rapid interconversion proves weak or absent base to base attractions and a low potential barrier. Thus it is contradictory to the two-state assumption which implicitly assumes the exclusion of intermediate states via a potential wall.
- Fluorescence polarization of NADH indicates that DHN rotates as a unit with the rest of the molecule. This is a good argument for a negligible share of the unfolded state.

Our measurements of absorption and fluorescence are incompatible with the existence of the two states.

- FRET quantum yield of NADH is near 50% under standard conditions. The system is easily disturbed by temperature changes or by guanidinium hydrochloride (GuHCl). But changes are moderate and an extreme state with 0% or 100% FRET is not found. Earlier claims of zero FRET under denaturing conditions seem to be due to an overestimation of direct excitation of DHN at 260 nm.
- The fluorescence spectrum of NADH is independent of direct excitation or excitation through FRET. There is no sign of an exciplex formation. This is in line with the identical emission spectra of NMNH and NADH found earlier and clearly contradicts a selective excitation of a folded state through the adenine.
- DHN absorption shows a small redshift and a slight decrease in absorption coefficient from NMNH to NADH but the spectral bandwidth stays unchanged. This is hardly compatible with any mixture of two states, especially not with base stacking and ring puckering.

We conclude that NADH in solution does not exist as an equilibrium of two extreme forms but as a single state.

Folding of the backbone leads to an ensemble of compact molecules with sufficient proximity for FRET. The potential curve is a well with a minimum around the Förster radius of 0.6 nm. Spectral differences between NMNH and NADH are low because the bases in the dinucleotide are separated by some solvent keeping their environment mainly aqueous. Chaotropic agents, cosolvents or temperature increase lead to increased flexibility and/or distance thereby diminishing FRET and NMR resonance shifts.

Introduction

In 1957, Weber (1) described the fluorescence of dihydronicotinamide (DHN) in NADH on excitation of adenine at 260 nm. He concluded that the fluorescence resonance energy transfer (FRET) would require an intimate contact of DHN and adenine. Fluorescence polarization indicated that DHN rotates as a unit with the rest of the molecule (2).

But FRET efficiency was ~30% only. FRET was studied on NADH and simplified synthetic compounds (3). Denaturation with methanol was used in 2001 to revisit the case (4). In both studies, data were explained by folded and open forms with high and low FRET. Spectroscopic work on NADH was generally analyzed using the folded/unfolded model. Solvent perturbation showed that the accessibility of both adenine and DHN to D₂O and sucrose was lower in NADH than in NMNH + adenine (5). Denaturants like methanol, urea or guanidinium promoted accessibility. The authors concluded that the adenine ring is stacked to the DHN moiety in the folded form. A study on fluorescence lifetime (6) explained the two lifetimes of NADH fluorescence via an exciplex of excited DHN with adenine in the folded form. A lot of NMR studies has been undertaken to clarify the solution structure of pyridine dinucleotides. Interaction of adenine with dihydronicotinamide (DHN) was shown, but not with oxidized nicotinamide (7). Sharp proton resonances indicated an equilibration rate below the ns timescale of NMR (7). Oppenheimer (8) claimed that DHN interaction with adenine would lead to base stacking and ring puckering. The cyano-adduct of nicotinamide adenine dinucleotide (NAD⁺) was used to demonstrate asymmetric interaction with adenine (8). A comparison of acidity constants of adenine in NAD⁺ and adenosine diphospho ribose (ADPR) was interpreted to show 44% folded form in aqueous solution (9). Studies were reviewed in 1982 (10, 11) and the authors explained that the folded/unfolded equilibrium is characteristic for all dinucleotides. The equilibrium was claimed to contain significant fractions of both forms irrespective of nicotinamide oxidation and excitation state. The folded state was concluded to contain stacked bases. In the unfolded state, the bases were assumed to be in an environment similar to that in AMP and NMNH. The case seemed clear in 1982. But there were and are conflicting views: The focussing of most studies to a folded/unfolded equilibrium as the single option for interpretation has been criticized early (12). NMR data on temperature dependency were incompatible with a simple two-state-model (7). Large variations in results between independent studies have to be noted as well: Hull mentioned a wide variation in the shares of the folded form of NADH (4). The same is true for the FRET efficiency in aqueous solution at 25°C: Values range from 30% (1,2) to 67% (4, 13). The Förster radius (dependent on the orientation of bases) was calculated to be around 0.6 nm (14) meaning that an intimate contact would not be necessary for efficient FRET. 2D-IR found weak interactions between adenine and DHN, incompatible with stacked bases (15). Summing up, the necessity of stacked bases inferred from FRET (2-4) is questionable. Fast dissociation, independency on the type of pyridine base and largely varying equilibrium results shed doubts on the two-state-model. In this study, we use absorbance and fluorescence spectroscopy to revisit the case.

Materials and Methods

Tris, NaCl, ammonium sulfate and guanidinium hydrochloride were of the highest available purity from Roth. NADH disodium salt grade 1 (100%) and NAD⁺ free acid grade 1 (100%) were purchased from Roche. AMP >99% and quinine sulfate >98% were from Sigma. NMN was a product of Cayman Chemical. Dansyl glycine was purchased from TCI. Sodium dithionite was a product of VWR.

The standard buffer was 0.1 M Tris/HCl, 0.1 M NaCl pH 8.5.

NMNH was prepared by mixing 5 mg NMN with 5 mg sodium carbonate and 5 mg sodium dithionite in 1 ml 1% sodium hydrogen carbonate buffer. After 5 min reaction time, 100 µl aliquots were purified through HPLC on a Phenomenex Synergi Fusion RP 18 column in 0.1 M ammonium acetate buffer. Under isocratic conditions, the main peaks (retention time 12.5 min) were collected and directly diluted into standard buffer to prepare 10⁻⁴ M and 10⁻⁵ M solutions for absorption and fluorescence measurements.

Absorption was measured on a Genesys 10S UV-Vis Spectrophotometer (Thermo Fischer Scientific). Rotilabo single use semi-micro plastic cuvettes with UV transparency were used for measurement series, e.g. GuHCl concentration dependency. 10 · 10 mm Quartz cells were used to measure absorption values and high precision spectra. Fluorescence was measured using a Cary Eclipse equipped with multi cell holder and temperature controller. Square (10 · 10 mm) quartz cells from Starna were used throughout

Results and Discussion

Structures of Nicotinamide Bases

Nicotinamides are hydrophilic molecules with dipolar structure, partial or full positive charge at N_1 and hydrogen bridges from and to the exocyclic group. Electronic structure and conformation depend on oxidation and excitation state (figure 1).

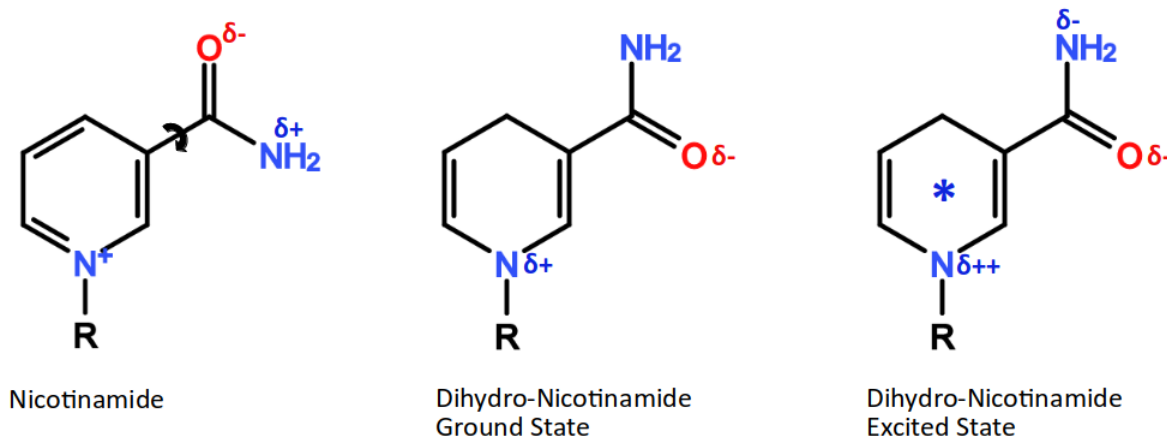


Figure 1: Electronic structures of nicotinamides

In Nicotinamide, the pyridinium moiety is positively charged. The external amide group is not conjugated, rotates freely and forms hydrogen bridges with donors and acceptors. Dihydro nicotinamide is a vinylogous amide. The exocyclic group is fixed in the ring plane with nitrogens in transoid position. Hydrogen bridges to donors and acceptors are formed (16). Excitation of dihydro nicotinamide shifts charge from N_1 to the exocyclic nitrogen, thereby weakening hydrogen bonds to acceptors. Donor hydrogen bridges to the carbonyl and thus the partial charge at the oxygen stay unchanged (16). If an attractive interaction of nicotinamides with adenine would be the driving force for base stacking, one would expect significant differences between the diverse types of nicotinamide bases. This is even more true for the cyano adduct of NAD^+ . Lack of differentiation is most easily understood by weak or absent binding. This is full in line with the fast dissociation and equilibration inferred from sharp NMR resonances ($k_d > 10^9/s$) (7).

Absorption Coefficients of Mono- and Dinucleotides

Absorption coefficients were taken from literature and complemented with own measurements as indicated in table 1.

Table 1: Absorption coefficients of nucleotides

Substance	λ : nm	ϵ : l · M ⁻¹ · cm ⁻¹	Reference
NMN	266	4200	17
NMN	260	3870	From spectrum normalized to ϵ_{max}
NAD ⁺	260	17400	18
NADH	260	14100	18
	340	6220	18
AMP	259	15080	19
	260	15060	19
NMNH	260	500	From spectrum normalized to ϵ_{max}
	340	6500	20

The absorption coefficient of NMNH at 260 nm is considerably lower than estimated from the spectrum of enzymatically hydrolyzed NADH (1,2). This eventually leads to a much lower contribution of direct DHN excitation to the fluorescence of NADH excited at 260 nm and to a higher FRET quantum yield (see below).

Table 2 shows a comparison of the absorption coefficients of mono- and dinucleotides in the adenine and nicotinamide absorption bands.

Table 2: Absorption coefficient comparison between mono- and dinucleotides

Substance	λ : nm	ϵ : l · M ⁻¹ · cm ⁻¹	$\Sigma \epsilon$: l · M ⁻¹ · cm ⁻¹	$\Delta \epsilon$: l · M ⁻¹ · cm ⁻¹
AMP + NMN	260	15060 + 3870	18930	
NAD ⁺	260		17400	-1530
AMP + NMNH	260	15060 + 500	15500	
NADH	260		14100	-1400
NMNH	340		6500	
NADH	340		6220	-280

The absorption of both dinucleotides is lower than the sum of mononucleotide absorptions both in the adenine and the nicotinamide absorption bands. Absorption coefficients demonstrate a change in environment of the chromophores between mono- and dinucleotides but a lot of different explanations is possible. The changes in the adenine chromophore have been found earlier to be rather unspecific (21).

Absorption Spectra of Dihyronicotinamides

Absorption spectra of NADH and NMNH are very similar at wavelengths >300 nm, where only DHN contributes. The absorption coefficient is slightly lower in NADH (6220 vs. 6500 l M⁻¹ cm⁻¹) and the maximum is shifted from 337 to 339 nm (cf. (5)). The folded/unfolded model assumes that DHN in unfolded NADH is fully exposed to solvent and thus similar to DHN in NMNH. The mean spectral shift of 2 nm then should be due to a mixture of unshifted and significantly shifted components. Attempts to compose the spectrum of NADH this way always led to spectral broadening. A comparison of the experimental spectra clearly shows that this is not the case (figure 4)

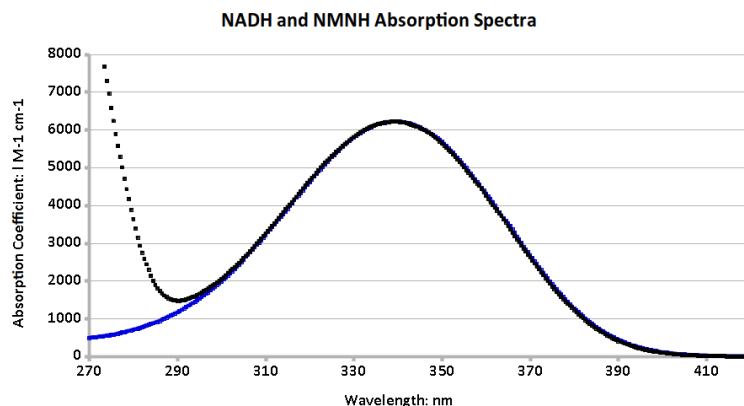


Figure 2: Normalized absorption spectra of NADH (black) and NMNH (blue)

The spectrum of NMNH was red-shifted by 2 nm. Both spectra were normalized to $\epsilon_{max} = 6220$ l M⁻¹ cm⁻¹.

We conclude that the vibrational sub-bands of DHN stay unchanged between NADH and NMNH and a very similar diversity of the solvent shell around the chromophore leads to effective broadening into the structure-less bands seen. A mixture of an NMNH-like state with a base-stacked state should lead to much larger differences. More strictly speaking, the absorption spectrum of NADH is incompatible with any mixture of two states, except with identical spectra. Apparently, we face a single state which is neither completely NMNH-like nor base stacked. This is full in line with 2D-IR results where weak interactions of bases were found (15).

Adenine Absorption

A comparison of normalized spectra shows that the half-width of the absorption band is increased by 0.5 - 1 nm on the red side in NADH (figure 3).

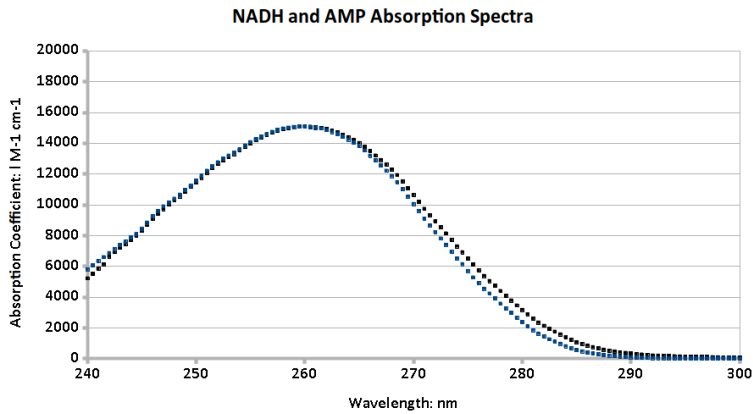


Figure 3: Comparison of Adenine Absorption Spectra in NADH (black) and AMP (blue).

The normalized spectrum of NMNH (cf. figure 2) was subtracted from the spectrum of NADH and both spectra were normalized to $\epsilon_{max} = 15060 \text{ l M}^{-1} \text{ cm}^{-1}$ at 260 nm.

This difference between the mono- and dinucleotide was interpreted earlier as a slight change in adenine protonation (5). A contribution of a base stacked state would be expected to lead to more significant changes.

Fluorescence Intensity

Fluorescence has been claimed to be concentration-dependent above 1 μM (4). This could be verified (figure 4).

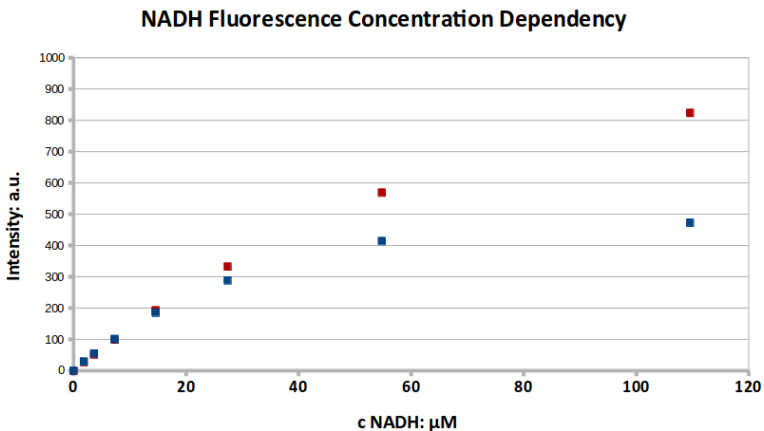


Figure 4: Concentration dependency of NADH fluorescence

red: Excitation at 340 nm

blue: Excitation at 260 nm

But a plot of intensity vs. the absorbed excitation light i.e. vs. (1-transmission) gave linear correlations for both excitation wavelengths, demonstrating that the concentration dependency is no more than the inner filter effect. Specific intensities (I/c) for both excitation wavelengths were extrapolated to zero concentration. The correction factor then was calculated by division of the specific intensities at a given concentration through this extrapolated value. Values for both excitation wavelengths follow the same linear correlation when plotted against the absorption (figure 5). This correlation was used to correct for the inner filter effect, e.g. in calculating the FRET quantum yield.

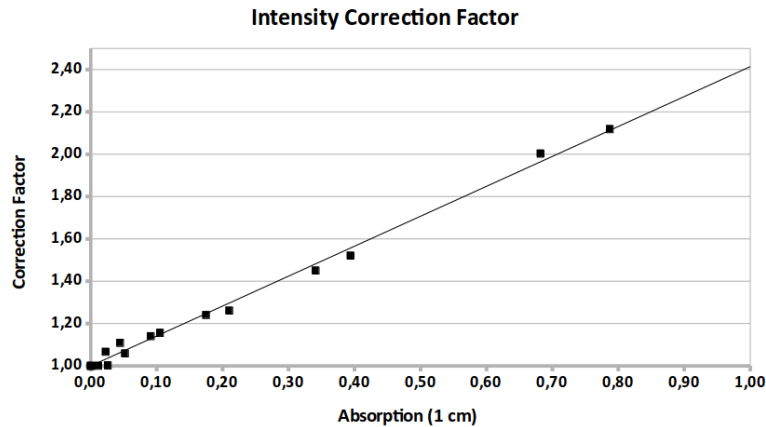


Figure 5: Correction factor for the inner filter effect.

Fluorescence intensity of NADH is independent of concentration up to at least 100 μM if the inner filter effect is taken into account. Our measurements were done mostly at 10 μM ($E_{340} = 0.062$) and corrected for the inner filter effect if necessary.

Spectra with direct and indirect excitation of DHN

Fluorescence intensity of NADH decreases with temperature concomitant with a slight red shift. But the normalized emission spectra are independent of excitation wavelength at all temperatures (Figure 6).

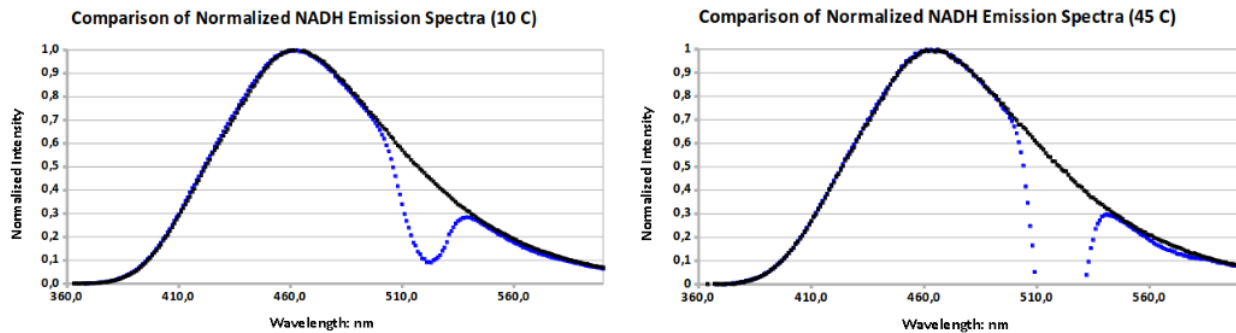


Figure 6: Normalized emission spectra of NADH with excitation at 340 nm (black) and 260 nm (blue).

The depression at around 520 nm is due to scattered 260 nm excitation (2nd order diffraction). The slight depression at around 560 nm is the corresponding Raman band. Due to the inner filter effect in the substance cuvette, blank subtraction leads to overcompensation. Scattering is independent of temperature but seemingly enhanced at higher temperature due to the normalization of the lowered fluorescence intensity.

In the folded/unfolded model, excitation at 260 nm is thought to excite the folded species only. The emission is identical to the emission after direct excitation of both folded and unfolded states at 340 nm, however. In the framework of the two-state model, both species would need to have identical emission spectra. The same has to be concluded comparing the normalized emission spectra of NADH and NMNH. They are superimposable (13). This is hardly conceivable for a folded state with base stacking. One might argue that the excited NADH unfolds to the NMNH-like state within <0.1 ns. So the spectrum always would be that of the unfolded state. But then the quantum yields of NADH and NMNH should be the same. One might argue, that ring puckering is the mechanism of radiationless deactivation (22), so the puckered ring in the folded state would not emit. But the quantum yield of NADH is higher than that of NMNH. Thus the unfolded state of NADH would need a high quantum yield to overcompensate the quenching of the folded state. This clearly contradicts the postulate of an unfolded state similar to NMNH. The assumption of a single state without base stacking would solve all these issues.

Fluorescence Lifetimes

Fluorescence lifetimes of NADH are independent of the wavelength of excitation (3). This is valid even for the two lifetimes of the non-monoexponential intensity decay (13). We have to conclude the same as from the spectra: A single state is emitting. The two lifetimes cannot be used as an argument for interaction with adenine (6) since NADH bound to ADH in stretched form with the bases far apart from each other shows two lifetimes (23) and NMNH decay is non-monoexponential as well (13). The two lifetimes are an intrinsic property of the DHN chromophore (24).

FRET

The classical argument (1) for stacked bases in NADH is the energy transfer on excitation of adenine at 260 nm. The non-linear intensity decrease with raising GuHCl is indeed an exception. Similar effects are seen in urea, methanol (4) and other cosolvents (10,21). Other fluorophores or NADH excited at 340 nm follow linear trend lines (figure 7).

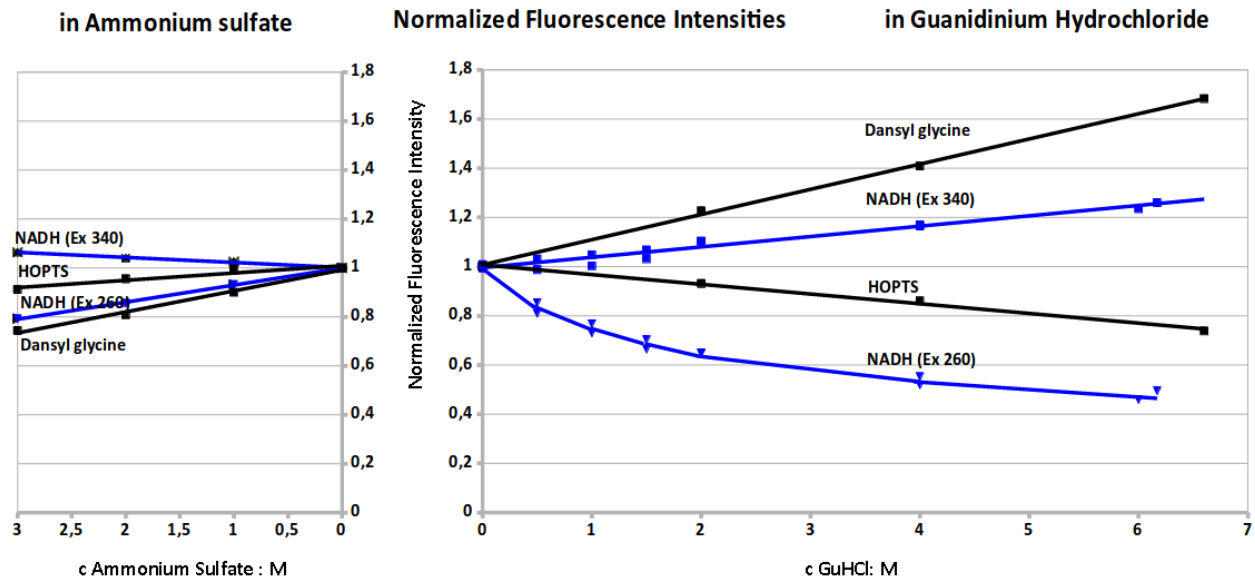


Figure 7: Normalized fluorescence intensities (emission at 460 nm)

HOPTS = Hydroxy pyrene trisulfonate

The NADH system is disturbed even by small concentrations of GuHCl. But this becomes visible only with excitation at 260 nm, not at 340 nm. In terms of the folded/unfolded model, decrease of FRET is interpreted as denaturation of the folded state. The sensitivity then is a sign of an equilibrium position near 1:1. If the system is so easily disturbed by a denaturing agent, one would expect that it should be shifted to the folded side and FRET might increase in ammonium sulfate. This is not the case. Instead a linear decrease of intensity to 80% at 3 M is found (figure 7, left). Excitation at 340 nm shows a further issue for the assumption of stacked bases: The DHN fluorophore shows the typical linear trend of other fluorophores without a special response to the postulated dissociation.

The measured lifetimes of excited DHN are the same irrespective of the excitation wavelength (3, 13). Thus the quantum yields are all the same once DHN is excited.

$$Q_{\text{DHN}(260)} = Q_{\text{DHN}(340)} = Q_{\text{NADH}(340)}$$

$$\text{and } Q_{\text{NADH}(260)} = Q_{\text{DHN}(260)} \cdot Q_{\text{FRET}} = Q_{\text{NADH}(340)} \cdot Q_{\text{FRET}}$$

$$\text{thus } Q_{\text{NADH}(260)} / Q_{\text{NADH}(340)} = Q_{\text{FRET}}$$

The normalized Q_{FRET} can be obtained by a simple division of the intensities at 260 and 340 nm (figure 8).

FRET follows a saturation curve with $Q_{\text{FRET min}} = 0.217$ and $c_{\text{GuHCl } 1/2} = 1.81 \text{ M}$. Fluorescence of NMNH resulting from direct absorption of 260 nm is much lower since the absorption coefficient is nearly 30fold lower than that of NADH (table 1).

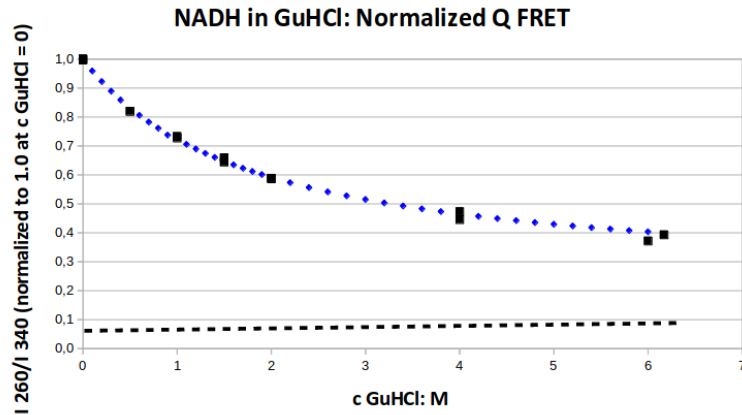


Figure 8: FRET in GuHCl

NADH excitations at 260 and 340 nm were used. Emission was observed at 460 nm.

Black squares: Measured data. Blue dots: Fit line.

Black broken line: Linear fit of NMNH fluorescence normalized to $I_{\text{NADH}(340)}$ and corrected for higher quantum yield of DHN in NADH (see "FRET efficiency" below).

The normalized intensity ratio stays significantly higher than that of NMNH. The decrease of FRET with denaturation does not lead to an NMNH-like state without FRET.

Temperature Dependency of Fluorescence

Temperature was varied between 45.5 and 1.1^o C. Stability at hot temperatures was checked repeatedly by cooling down to 25^o C. Intensities were found unchanged. Intensities shown in figure 9 are normalized to the values at 25^o C.

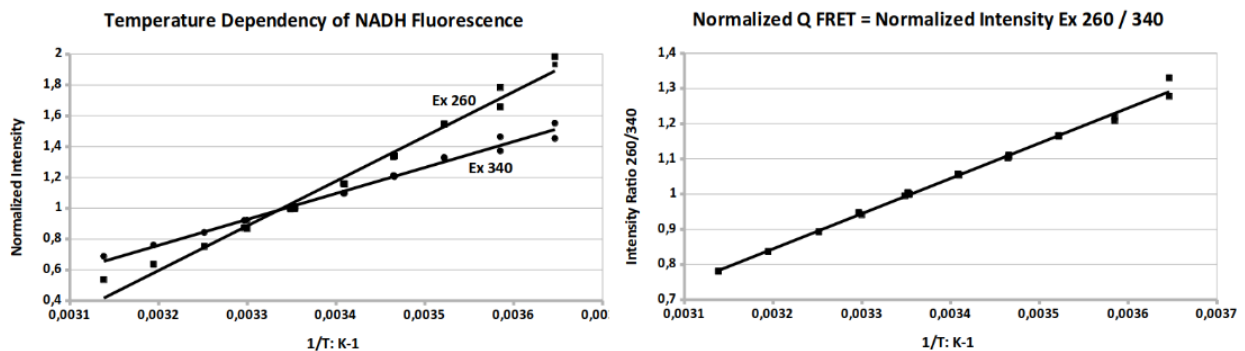


Figure 9: Temperature dependency of normalized intensities and FRET efficiency

In line with earlier data (4), fluorescence intensity decreases with increasing temperature. The slope is higher with indirect excitation at 260 nm (figure 9, left). The Q_{FRET} curve is linear (figure 9, right) with a slope slightly lower than that of NADH at 340 nm excitation. Like in the case of GuHCl, the intensities change with small changes without any lag phase. An S-curve indicating saturation at one or the other end is not seen. The system is easily disturbed by temperature and does not reach a limiting state on either side within the measurement range.

FRET efficiency

Quantum yields of NADH at 260 nm were first determined using the well known comparison with quinine sulfate. Values of 0.85 – 1.05 % were obtained, slightly lower than the 1.1% found earlier (13). Dividing by the quantum yield of 1.6% with excitation of 340 nm, we get Q_{FRET} values of 49 to 59%. The method is not without problems: It has to deal with the more than 50fold lower intensity of NADH and great care has to be taken to correct for the blank overcompensation at >500 nm (cf. figure 6). In addition, the steep slope of absorption around 260 nm in

quinine sulfate might lead to an absorption bias towards the blue side and correspondingly to higher absorption. We used the minimal slit width of 1 nm to mitigate the effect.

Additionally we used a direct comparison of NADH with NMNH.

- Approximately 10^{-5} M solutions of NADH and NMNH with matching absorptions of 0.062 at 340 nm were used. Since the emission spectra are exactly the same, the intensity ratio (excitation 340 nm, emission at 460 nm) is directly proportional to the quantum yield ratio. The result was $Q_{\text{NADH}(340)} / Q_{\text{NMNH}(340)} = 1.33$.

- All intensities were corrected for the inner filter effect (see figure 5).

- The intensity of NMNH with excitation at 260 nm was multiplied by 1.33 to account for the higher quantum yield of DHN in the dinucleotide and subtracted from the intensity of NADH. This was a 6% correction only. Weber (1,2) and Scott (3) apparently used a much higher correction eventually leading to a lower FRET efficiency.

- The absorption of NMNH at 260 nm was directly subtracted from the absorption of NADH. This was a correction of -3%. So the question of a slightly deviating absorption coefficient of DHN in NADH at 260 nm could be neglected.

- All intensities were divided by the corresponding absorptions i.e. they were normalized to $E = 1$.

- The ratio values of the normalized intensities I_{260} / I_{340} were 2.2 for NMNH and 1.14 for NADH. The ratio for NADH is lower, since FRET lowers the intensity at 260 nm excitation by the factor $Q_{\text{FRET}} = 0.52$. This is not a highly precise result either since small errors in absorption of NADH and especially NMNH at 260 nm are enlarged by the normalization. But the agreement with the quinine sulfate based values is satisfactory.

The FRET efficiency of 50-60% under standard conditions is well in line with the results of temperature perturbation. Linear response without reaching end points would not be expected at 75 or 25%.

Förster radius

Decreasing FRET with increasing GuHCl (figures 7,8) or increasing temperature (figure 9) is certainly not a proof of the folded/unfolded model. The experiments show only that the mean distance and/or the orientation of the bases change. The connection to base stacking came from the postulate of Weber that FRET would require an intimate contact (1). Adenine fluorescence has been measured meanwhile. The mean lifetime is ~ 300 ps and quantum yield is $5.6 \cdot 10^{-5}$ (25). A calculation of the Förster radius gave values around 0.6 nm dependent on the orientation factor κ^2 (14). These results allow FRET with some distance between the bases.

Indeed, FRET without base stacking has been experimentally shown as early as 1970. Scott (3) compared NADH with simplified model compounds where adenine and DHN were linked by a hexyl and a propyl chain. All compounds showed FRET in aqueous solution. NADH and the hexyl compound lost FRET in propylene glycol and the explanation was that propylene glycol dissolved base stacking and folding. The propyl compound retained substantial FRET, however. The authors claimed that the short trimethylene bridge was responsible (3). Thus these experiments have demonstrated efficient FRET over the trimethylene distance of roughly 0.5 nm under denaturing conditions where base stacking was claimed to be absent. Webers qualitative conclusion (1) was not justified. An intimate contact is not necessary for FRET.

Conclusion

A model without intimate base to base contact is proposed. It is characterized by a stereospecific folding of the backbone but not by base stacking and not by a two-state folded/unfolded equilibrium. NADH is a compact molecule in aqueous solution at ambient temperature. Bases are separated by solvent. Solvents like methanol or chaotropic agents like GuHCl and urea lead to enhanced flexibility. Orientation and/or distance between adenine and DHN change and FRET and NMR spectral shifts decrease accordingly. A similar change is caused by raising temperature.

Spectral changes in both chromophores are unspecific since they are due to a large degree to changing water concentration around the chromophores irrespective of the agent. A change in the aqueous solvent shell explains as well why the quantum yield of DHN in NADH is larger than in NMNH. Solvents like methanol, concentrated salts like GuHCl or the second chromophore adenine displace water slowing down the radiation-less deactivation of DHN. A mono- or bimolecular layer of water between the bases explains why the differences in spectral properties between mono- and dinucleotides are small. But proximity is sufficient for FRET and NMR resonance shifts.

The new model replaces the two extremely different completely folded and unfolded states by a single state: an ensemble of compact but flexible folded structures. Transitions between short-lived conformations need small

intramolecular motions only instead of complete folding/unfolding with base stacking and unstacking. Thus rapid interconversions below the timescale of NMR and gradual changes responding to environment changes without signs of saturation at one or the other end are a natural consequence. Assuming that backbone folding has a stereospecific selectivity preferentially bringing one side of the DHN ring closer to the adenine, the model is compatible with the observed stereospecific chemical reactivity and differentiated NMR signals of the C_4 protons of DHN (11). These compact NADH molecules rotate as units without significant relative mobility of DHN as indicated by fluorescence polarization (2).

The implicit thermodynamic problem of the folded/unfolded two state model is avoided. Two states require a high energy barrier between them to suppress the many intermediate conformations of the flexible molecule. But fast interconversion with rate constants $k > 10^9/s$ needs a low one (figure 10, left). The potential wall of the old model is replaced in the new one by a potential well, which is slightly changed with solvent or temperature changes. Reactions back and forth between a little bit more or less compact structures are fast because potential walls are absent (figure 10, right).

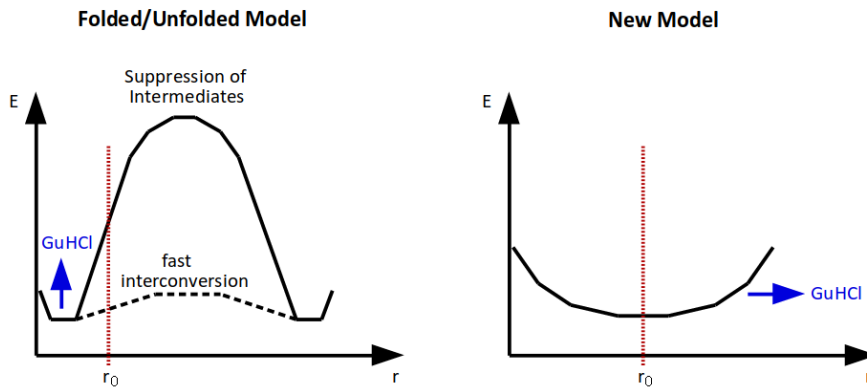


Figure 10: Two-state model and new model
 r_0 is the Förster radius.

The model integrates the idea of McDonald that NMR resonances „...could be different in different environments if different minimum intramolecular distances between the nicotinamide and the adenine rings constitute what we refer to as the completely folded conformer.“(7). In addition, the new model eliminates the completely unfolded conformer. It is not necessary to explain FRET and NMR data, a significant NMNH-like contribution to absorption is absent and fluorescence polarization indicates that DHN does not rotate relative to the rest of the molecule (2) excluding a significant share of the unfolded state. Elimination of the base stacked state is consistent with the IR data showing weak base to base interactions (15).

References

- (1) Weber,G., "Intramolecular transfer of electronic energy in dihydro diphosphopyridine dinucleotide", *Nature* 180, p. 1409 (1957)
- (2) Weber,G., „Transfert d'énergie dans la dihydro-diphosphopyridine dinucleotide“, *Journal de chimie physique et de physicochimie biologique* 55, pp.878-886 (1958)
- (3) Scott,T.G., Spencer,R.D., Leonard,N.J., Weber,G., "Emission properties of NADH. Studies of fluorescence lifetimes and quantum efficiencies of NADH, AcPyADH, and simplified synthetic models," *J. Am. Chem. Soc.*, 92, pp. 687–695 (1970)
- (4) Hull,R.V., Conger,P.S.III, Hoobler,R.J. "Conformation of NADH studied by fluorescence excitation transfer spectroscopy", *Biophysical Chemistry* 90, pp. 9-16 (2001)
- (5) Cross,D.G., Fisher,H., „Conformation and Conformational Changes of Reduced Diphosphopyridine Nucleotide in Solution“ *Biochemistry* 8,3, pp.1147-1155 (1969)
- (6) Visser,A.J.W., van Hoek,A., "The Fluorescence Decay of Reduced Nicotinamides in Aqueous Solution after Excitation with a UV-Mode Locked Ar Ion Laser", *Photochemistry and Photobiology* 33, pp.35-40 (1981)
- (7) McDonald,G., Brown,B.,Hollis,D.,Walter,C., „Some Effects of Environment on the Folding of Nicotinamide-Adenine Dinucleotide in Aqueous Solution“ *Biochemistry* 11, pp. 1920-1930 (1972)
- (8) Oppenheimer,J.O., Arnold,L.J., Kaplan,N.O., "A Structure of Pyridine Nucleotides in Solution" *Proc. Natl. Acad. Sci. USA*, 68(12), pp. 3200–3205 (1971)
- (9) Sovago,I.,Martin,R.B. „Nicotinamide adenine dinucleotide is about 44% folded in neutral aqueous solution“, *FEBS letters* 106, pp. 132-134 (1979)
- (10) Ross,J.B.A., Subramanian,S., Brand,L. „Spectroscopic Studies of the Pyridine Nucleotide Coenzymes and Their Complexes with Dehydrogenases“ pp. 19-49 in „The Pyridine Nucleotide Coenzymes“, Everse,J., Anderson,B., You,K.-S. Eds. Academic Press ISBN 978-0-12-244750-1
- (11) Oppenheimer,N.J. „Chemistry and Solution Conformation of the Pyridine Coenzymes“ pp. 51-89 in „The Pyridine Nucleotide Coenzymes“, Everse,J., Anderson,B., You,K.-S. Eds. Academic Press ISBN 978-0-12-244750-1
- (12) Jacobus,J. „Conformation of Pyridine Dinucleotides in Solution“, *Biochemistry* 10, pp. 161-164 (1971)
- (13) Von Ketteler, A., „Fluoreszenzspektroskopie von carba-Dihydronicotinamidadenin-dinukleotid“, Dissertation, Heidelberg (2012)
- (14) Heiner,Z., Roland,T., Leonard,J., Haake,S., Groma,G., "Kinetics of Light-Induced Intramolecular Energy Transfer in Different Conformational States of NADH", *J. Phys. Chem. B*, 121, pp. 8037-8045 (2017)
- (15) Simpson,N. et al, "Infrared Spectroscopy of Nicotinamide Adenine Dinucleotides in One and Two Dimensions" *J. Phys. Chem. B*, 117, 16468–16478 (2013)
- (16) Fischer,P., Fleckenstein,J., Hönes,J., „Spectroscopic investigation of Dihydronicotinamides I: Conformation, absorption and fluorescence“, *Photochem. and Photobiol.* 47,2, pp.193-199 (1988)
- (17) Liu,R.,Visscher,J. „A Novel Preparation of Nicotinamide Mononucleotide“ *Nucleosides, Nucleotides & Nucleic Acids* 13:5, pp.1215-1216 (1994)
- (18) Hald,E.,Lehmann,P.,Ziegenhorn,J. „Molar Absorptivities of β -NADH and β -NAD at 260 nm“, *Clin.Chem.* 21/7, pp.884-887 (1975)
- (19) Cavaluzzi,M.J., Borer,P.N., „Revised UV extinction coefficients for nucleoside-5' monophosphates and unpaired DNA and RNA“, *Nucl. Acid Res.* 32 No. 1 e13 (2004)
- (20) Baumgarten,B., „Wechselwirkungen in Binär- und Ternärkomplexen der Malat- und Lactat-Dehydrogenase“ Dissertation Universität Stuttgart (1989)

- (21) Subramanian,S., Ross,J.B., Ross,P.D., Brand,L. "Investigation of the nature of enzyme-coenzyme interactions in binary and ternary complexes of liver alcohol dehydrogenase with coenzymes, co-enzyme analogues, and substrat analogues by ultraviolet absorption and phosphorescence spectroscopy" *Biochemistry* 20, pp. 4086-4093 (1981)
- (22) Blacker,T.S., Marsh,R.J., Duchen,M.R., Bain,A.J., "Activated barrier crossing dynamics in the non-radiative decay of NADH and NADPH", *Chemical Physics* 422, pp.184-194 (2013)
- (23) Gafni,A., Brand,L., "Fluorescence Decay Studies of Reduced Nicotinamide Adenine Dinucleotide in Solution and Bound to Liver Alcohol Dehydrogenase", *Biochemistry*, vol. 25, pp. 3165–3171 (1976)
- (24) Krishnamoorthy,G., Periasamy,N., Venkataraman,B., "The Origin of Heterogeneity of Fluorescence Decay Kinetics of Reduced Nicotinamide Adenine Dinucleotide", *Biochem. Biophys. Res. Comm.*, vol. 144, pp. 387–392 (1987)
- (25) Kwok,W.-M., Ma,C., Phillips,D.L., "Femtosecond Time- and Wavelength-Resolved Fluorescence and Absorption Spectroscopic Studies of the Excited States of Adenosine and an Adenine Oligomer", *J.Am.Chem.Soc.* 128, pp.11894-11905 (2006)