Correlation of Electronic Effects in *N*-Alkylnicotinamides with NMR Chemical Shifts and Hydride Transfer Reactivity

James R. Burke† and Perry A. Frey*

Institute for Enzyme Research, The Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53705

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The ^{13}C and ^{15}N NMR chemical shifts for ring atoms of a series of *N*-alkylnicotinamides are shown to be correlated with their reduction potentials and reactivities toward $NaBH_3CN$. The nicotinamide compounds include *N*-ethyl-*N*-benzyl-*N*-[*p*-(trifluoromethyl)benzyl]-, *N*-(*p*-cyanobenzyl)-, *N*-(carbomethoxymethyl)-, and *N*-(cyanomethyl)nicotinamides. The values of $\delta_{^{13}C}$ for all the ring carbons increase with increasing electron-withdrawing power of the *N*-alkyl substituent. The value for C-4 increases the most, a range of 2.4 ppm in this series, whereas those for other atoms increase on the order of 1 ppm. The value of $\delta_{^{15}N}$ for N-1 decreases with increasing electron-withdrawing power over a range of 20 ppm. The NMR data indicate that inductive electron withdrawal by *N*-alkyl substituents polarizes the π-electron system to decrease electron density on ring carbons and increase electron density on the ring nitrogen. The values of log *k* (second order) for reduction of these compounds by $NaBH_3CN$ are proportional to the values of $\delta_{^{13}C}$ for C-4 and inversely proportional to $\delta_{^{15}N}$ for N-1. The reduction potentials are proportional to $\delta_{^{13}C}$. The substituent effects are qualitatively similar to the substrate-induced electrostatic effects on the nicotinamide ring of NAD^+ at the active site of UDP-galactose 4-epimerase (Burke, J. R.; Frey, P. A. *Biochemistry* **1993**, *32*, 13220−13230). However, they differ quantitatively in that the upfield perturbation at N-1 is smaller in the enzyme and the signal for C-6 is also shifted upfield. The substrate-induced enzymatic perturbation of electron density at C-4 of NAD^+ quantitatively accounts for its increase in reactivity at the active site, but the perturbation at N-1 is less closely correlated with reactivity.

Pyridine nucleotide dependent oxidoreductases catalyze the transfer of a hydride equivalent between the nicotinamide ring of NAD^+ and an alcohol (eq 1).

$$NAD^+ + RCH_2OH \rightleftharpoons 1,4\text{-NADH} + RCHO + H^+ \quad (1)$$

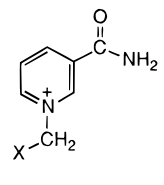
The reaction is thought to require general acid−base catalysis by an enzymatic group in the active site to facilitate the removal of the alcoholic proton at neutral pH. This would account in part for enzymatic catalysis of the reaction of an alcohol in the forward direction (and an aldehyde in reverse). However, general acid−base catalysis could not be brought to bear on NAD^+ itself to facilitate hydride transfer. The means by which an enzyme increases the chemical reactivity of NAD^+ is an interesting subject that is receiving renewed attention with the determination of the complete three-dimensional structures of NAD^+-dependent enzymes. In addition to X-ray crystallography, spectroscopic techniques are being brought into play to analyze the effects of enzymatic binding interactions on bonding and reactivity in the nicotinamide ring of NAD^+.[1,2]

In several cases, electrostatic interactions between the nicotinamide ring of NAD^+ and enzymatic groups have been invoked to explain catalysis of hydride transfer. Electrostatic repulsion between the 6-ammonium group of Lys[153] in UDP-galactose 4-epimerase and nicotinamide-N-1 of NAD^+ has been postulated to polarize the π-electron system of NAD^+, increasing positive charge on C-4 and enhancing its reactivity at the active site.[2,3] Interactions of C-4 with anionic and polar groups in other enzymes have also been postulated to increase its positive charge.[4,5]

We have undertaken the evaluation of the spectroscopic and chemical consequences of such electrostatic effects through studies of electronic effects on NMR chemical shifts and rates of reduction in a series of *N*-alkylnicotinamides. We find that through-bond electronic effects in these compounds can be correlated with the spectroscopic changes and rate acceleration observed for UDP-galactose 4-epimerase.

Results

The *N*-alkylnicotinamides **1a**−**1f** may be regarded as models of the nicotinamide ring in NAD^+ for the purposes of relating their spectroscopic properties to their chemical reactivities. Values of $\delta_{^{13}C}$ for carbons 2−6 of the nicotinamide ring for each compound in water, as assigned by two-dimensional $^{13}C-^1H$ correlation spectroscopy, are given in Table 1 together with $\delta_{^{15}N}$ for N-1 of the corresponding [1-^{15}N]-labeled compounds. Table 2 shows how these chemical shifts are affected when the NMR



X = CH_3	**1a**
Ph	**1b**
p-$PhCF_3$	**1c**
p-PhCN	**1d**
CO_2CH_3	**1e**
CN	**1f**

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Table 1. Values of ^{13}C and ^{15}N Chemical Shifts for the Nicotinamide Ring Carbons and Nitrogen of *N*-Alkylnicotinamides in Water

	chemical shifts (ppm)					
X (compd)	^{13}C-2	^{13}C-3	^{13}C-4	^{13}C-5	^{13}C-6	^{15}N-1
CH_3 (**1a**)	146.67	136.53	146.40[a]	130.99	148.83	219.31
Ph (**1b**)	146.93	136.70	146.93[a]	131.16	149.09	217.29
p-PhCF_3 (**1c**)	147.31	136.93	147.31	131.41	149.37	
p-PhCN (**1d**)	147.35	136.96	147.35[a]	131.42	149.46	213.95
CO_2CH_3 (**1e**)	148.62	136.59	147.93[a]	131.00	150.62	202.84
CN (**1f**)	147.83	137.17	148.83[a]	131.68	149.67	198.30

[a] Previously reported.[2]

Table 2. Values of ^{13}C and ^{15}N Chemical Shifts for the Nicotinamide Ring Carbons and Nitrogen of *N*-Alkylnicotinamides in Aqueous Ethanol[a]

	chemical shifts (ppm)					
X (compd)	^{13}C-2	^{13}C-3	^{13}C-4	^{13}C-5	^{13}C-6	^{15}N-1
CH_3 (**1a**)	146.19	136.54	145.98[b]	130.68	148.35	219.59
Ph (**1b**)	146.40	136.70	146.40[b]	130.89	148.51	218.38
p-PhCF_3 (**1c**)	146.84	137.01	146.84	131.25	148.84	
p-PhCN (**1d**)	146.82	136.91	146.82[b]	131.16	148.83	214.36
CO_2CH_3 (**1e**)	148.62	136.64	147.46[b]	130.73	150.46	203.83
CN (**1f**)	147.62	137.38	148.41[b]	131.58	149.30	199.39

[a] Measured in 86% ethanol. [b] Previously reported.[2]

measurements are carried out in 86% ethanol, a solvent that may be more similar than water to an enzymatic microenvironment. In both solvents, the electron-withdrawing substituents increase the shielding on N-1 and decrease the shielding on C-4. The effects on the other carbon atoms in the ring are much smaller than those on C-4, but in the same general direction.

The second-order rate constants for reduction of the *N*-substituted nicotinamide bromides by sodium cyanoborohydride in aqueous solutions and in 86% ethanol are given elsewhere. The rate constants increase in a logarithmic relationship with $\delta_{^{13}C\text{-}4}$, which is proportional to the electron-withdrawing power of the substituent, X. The linear plot of log k against $\delta_{^{13}C\text{-}4}$ for five of the compounds is shown elsewhere;[2] the earlier results are expanded here to include **1c**, for which $k = (0.99 \pm 0.30) \times 10^{-3}$ M^{-1} s^{-1} in water and $k = (17.0 \pm 1.7) \times 10^{-3}$ M^{-1} s^{-1} in 86% ethanol. The rate constants and the corresponding values of $\delta_{^{13}C4}$ for **1c** fit perfectly onto the linear plots of log k against $\delta_{^{13}C\text{-}4}$ published for the other five compounds.[2] The value of $E° = -383 \pm 7$ mV for **1c** also fits the published correlation of reduction potentials with $\delta_{^{13}C4}$.[2]

The proportionality of log k to $\delta_{^{13}C\text{-}4}$ is consistent with the nature of the reduction reaction, in which there is a significant increase in the electron density in the nicotinamide ring upon proceeding to the transition state from the cationic nicotinamide. The reduction of **1c** and other *N*-alkylnicotinamides proceeds more than 1 order of magnitude faster in 86% ethanol than in water. The solvent effect is also as expected because desolvation in ethanol would have a greater effect on the ground state than on the transition state, in which there is less charge separation. The larger substituent effects in 86% ethanol than in water are also explained by the differential solvation of ground and transition states.[2] Because the ground state is less strongly solvated in 86% ethanol than in water, the energy differences within the series of model compounds in both the ground state and transition state will have a proportionately larger effect on the activation energy in ethanol.

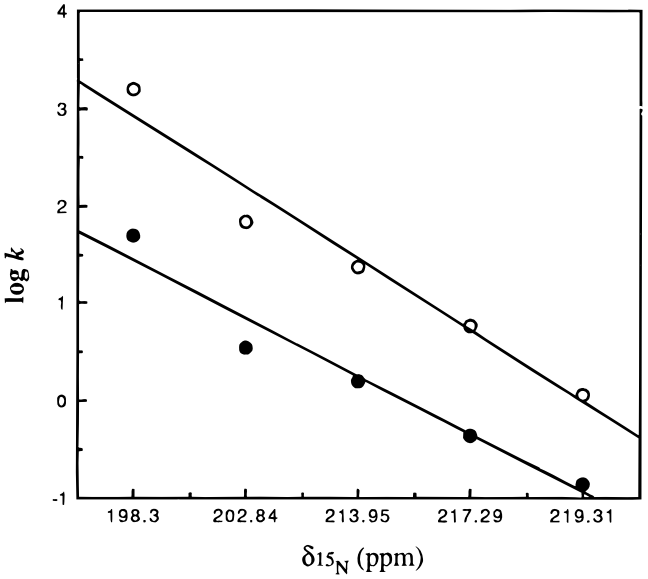

Figure 1. Plots of log k for reduction of *N*-alkylnicotinamides against $\delta_{^{15}N}$ in water and 86% ethanol. Shown are semilogarithmic plots of the second-order rate constants for the reduction of *N*-alkylnicotinamides against the values of $\delta_{^{15}N}$ for the same compounds (Tables 1 and 2) in water (filled circles) and 86% ethanol (open circles). The slopes are −0.17 (water) and −0.27 (86% ethanol).

Discussion

Correlations of NMR Chemical Shifts with Reactivities. There is a good semilogarithmic correlation between the second-order rate constants for the reduction of *N*-alkylnicotinamides with their values of $\delta_{^{13}C}$ at C-4, both in water and in 86% ethanol.[2] As the electron-withdrawing capacity of the substituent is increased, the value of $\delta_{^{13}C}$ at C-4 shifts downfield. Plots of log k versus $\delta_{^{13}C\text{-}4}$ exhibit slopes of 1.03 and 1.23 in water and 86% ethanol, respectively.[2] The electron density at C-4 decreases with increased electron withdrawal in the *N*-alkyl group, leading to decreased shielding at this carbon, which is correlated with increased reactivity.

With the exception of the values for **1e** (X = CO_2CH_3), analogous correlations are seen for $\delta_{^{13}C}$ at C-2, C-3, C-5, and C-6. However, the magnitudes of the chemical shift perturbations at these centers are smaller than at C-4. Indeed, the chemical shift differences between **1a** and **1f** at C-2 and C-6 in 86% ethanol are only 1.43 and 0.95 ppm, respectively, whereas for C-4 it is 2.43 ppm. The differences are only 0.84 and 0.90 ppm at C-3 and C-5, respectively.

Borohydrides reduce *N*-alkylnicotinamides to both 1,4-dihydro and 1,6-dihydro forms.[6] The ^{13}C data in Table 2 show that C-4 is much more susceptible to electron withdrawal than C-6. Therefore, while the observed rates must be regarded as composite for both reduction modes, most of the electronic effect is probably associated with rate effects on C-4.

There is also a linear correlation between log k and $\delta_{^{15}N1}$; however, the slope is negative, −0.17 in water and −0.27 in 86% ethanol, as illustrated in Figure 1. The value of $\delta_{^{15}N1}$ is moved progressively upfield as the substituent becomes more electron-withdrawing and corresponds to a difference of 20 ppm between **1a** and

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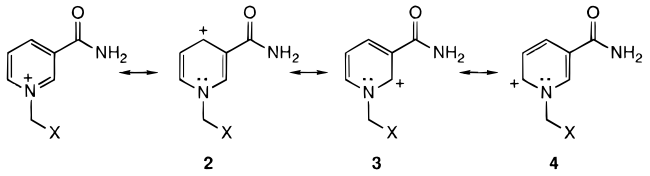
1f. This is consistent with an increase in electron density at N-1 as the electron-withdrawing capacity of the *N*-alkyl substituent increases, so that nitrogen becomes more shielded. These results indicate that electron-withdrawing *N*-alkyl substituents transfer electron density from ring carbons to N-1. Moreover, the fact that the changes in $\delta_{^{13}C}$ are largest at C-4 suggests that resonance form **2** is becoming more important than **3** or **4** as the electron-withdrawing capacity of the substituent increases.



The fact that the magnitude of the chemical shift changes at N-1 are much larger than at C-4 is consistent with the effect seen in monosubstituted benzenes, which showed the signals of the aromatic carbon atom bearing the substituent (C-1) to be affected more than the *para* aromatic carbon (C-4).[7] For example, upon going from toluene to α,α,α-trifluorotoluene, the change in the chemical shifts at C-1 and C-4 are 17.0 ppm upfield and 6.1 ppm downfield, respectively.

The NMR data clearly indicate that polarization of the π-electron system through electron withdrawal by *N*-alkyl substituents decreases electron density on the carbon atoms, principally at the para position (C-4), and increases the electron density at N-1.

Reduction Potentials. Two-electron reduction potentials for *N*-alkylnicotinamides were calculated by use of an indirect method, in which aqueous solutions of the nicotinamide salts were allowed to equilibrate with cyanide ion at 20 °C.[8] It has been shown that the addition of cyanide is reversible, it takes place at the 4-position, and the free energy changes associated with cyanide addition correspond well with the free energy changes of direct hydride transfer.[9] The equilibrium constant was determined by studying the equilibrium at different cyanide concentrations. Therefore, by comparing the equilibrium constant for reaction of cyanide with a model nicotinamide to that for NAD^+, for which the reduction potential is −348 mV versus the hydrogen electrode at 20 °C and pH 8,[10] the reduction potentials for the *N*-alkylnicotinamides can be approximated. Two-electron reduction potentials calculated using this cyanide affinity method have been shown to agree very well with those measured both enzymatically and potentiometrically.[9,11]

Correlation with Enzymatic Reactivity of NAD^+. The present results further substantiate the correlation between the rate of chemical reduction of NAD^+ bound at the active site of UDP-galactose 4-epimerase and the ^{13}C chemical shift at nicotinamide C-4. This enzyme is subject to a uridine nucleotide-induced conformational change that alters the chemical reactivity of NAD^+ bound to the active site. For example, the binding of uridine 5′-phosphate (UMP) or uridine 5′-diphosphate (UDP) by this enzyme strikingly increases the rate at which NAD^+ is reduced at the active site by glucose, $NaBH_4$, or $NaBH_3CN$.[12,13] Studies of the effects of UDP on the chemical shifts of the enzymatic complexes **E**·[4-^{13}C]NAD^+ and **E**·[1-^{15}N]NAD^+ show that UDP decreases shielding at C-4 and increases shielding at N-1, indicating that electron density has been withdrawn from C-4 by UDP-binding.[2] On the basis of the enzyme structure, ring current effects cannot be invoked to account for the NMR data.

The effect of the enzyme conformational change on the NMR properties of nicotinamide-C-4 and -N-1 in NAD^+ are qualitatively similar but not exactly the same as the effects of electron-withdrawing substituents in *N*-alkylnicotinamides. The electron-withdrawing substituents decrease shielding on all the ring carbon atoms of *N*-alkylnicotinamides while increasing the shielding of N-1. The effects of the enzyme conformational change are qualitatively analogous at C-4 and N-1 of enzyme-bound NAD^+, but the conformational effect differs in two respects. First, although the conformationally induced perturbation in $\delta_{^{13}C}$ at C-4 of NAD^+ at the active site (∼3 ppm) is in the range of the largest change observed in the model compounds (∼2.4 ppm), the enzymatic perturbation in $\delta_{^{15}N}$ at N-1 (∼3 ppm) is less than the maximum change in the model compounds (∼20 ppm). Second, the conformational change slightly decreases shielding at C-2 and slightly increases shielding at C-6 of NAD^+. Therefore, part of the increased electronic shielding in the enzymatic perturbation of NAD^+ is directed to N-1 and part to C-6, unlike electron withdrawal in the model compounds. This presumably arises from the fact that the enzymatic conformational effects on NAD^+ are not mediated by through-bond inductive effects but appear to be brought about by electrostatic repulsion between nicotinamide-N-1 of NAD^+ and the 6-ammonium ion of Lys^{153} in the protein.[3] Such a through-space electrostatic effect will not be centered on N-1 but may be directed along a slightly peripheral direction between N-1 and C-6 of NAD^+ bound to the active site. This means that the π-electron system of the nicotinamide ring in **E**·NAD^+·UDP is asymmetrically distorted, with increased electron density conferred on C-6 and N-1 at the expense of decreased electron density on other carbon atoms, mainly C-4.

UMP increases the reactivity of **E**·NAD^+ 500-fold with $NaBH_3CN$[3] and 3000-fold with glucose.[14] The enzymatic perturbation in $\delta_{^{13}C\text{-}4}$ is 2.8−3.4, depending on the nucleotide that induces it. Correlation of data in Tables 1 and 2 with reduction rates[2] shows that a 2.8−3.4 ppm downfield perturbation in $\delta_{^{13}C4}$ corresponds to an 800 to 3200-fold increase in reduction rate in water and a 2700−15 000-fold increase in 86% ethanol. Carbon-4 is the site of hydride transfer in NAD^+. We therefore conclude that the enhanced reactivity of NAD^+ induced by substrate binding is quantitatively correlated with the decreased electron density at C-4. Values of $\delta_{^{15}N}$ in the same model compounds range over 20 ppm, about seven times the substrate-induced enzymatic perturbation in NAD^+. The enzymatic perturbation at N-1 is only 2.6−3.0 ppm,

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depending on the nucleotide, and it is only qualitatively correlated with the increased reactivity of NAD^+.

Experimental Section

Materials. The syntheses of 1-ethylnicotinamide bromide (**1a**), 1-benzylnicotinamide bromide (**1b**), 1-[4-(trifluoromethyl)-benzyl]nicotinamide bromide (**1c**), 1-(4-cyanobenzyl)nicotinamide bromide (**1d**), 1-(carbomethoxymethyl)nicotinamide bromide (**1e**), and 1-(cyanomethyl)nicotinamide bromide (**1f**) were previously reported.[15−17] The *N*-alkylnicotinamide bromides labeled with ^{15}N at the N-1 position in the ring were prepared from [1-^{15}N]nicotinamide[18] by the same procedures used for the parent compounds.

Measurement of Rates and Reduction Potentials. The rates at which the *N*-alkylnicotinamides are reduced by $NaBH_3CN$ in aqueous solution and in 86% ethanol were measured as described elsewhere.[2] The reduction potentials were measured by the general method of Wallenfels and Diekmann,[8] as described elsewhere.[2]

NMR Spectroscopy. Proton-decoupled ^{13}C NMR spectra of 35 mM aqueous (50% D_2O) and 5 mM ethanolic (86% ethanol, 7% D_2O) solutions of each model nicotinamide at 25 °C were obtained at 100.6 MHz on a Bruker AM-400 spectrometer equipped with a 10 mm broadband probe. Values for ^{13}C chemical shifts were determined from the signal for the isotope at natural abundance and are reported as ppm downfield from 3-(trimethylsilyl)propanesulfonic acid, sodium salt, although they were referenced to external 10% dioxane in water (10% D_2O) which was assigned a shift of 69.22 ppm. In order to assign the ^{13}C NMR resonances accurately, two-dimensional $^{13}C-^1H$ correlation spectra of some of the aqueous model nicotinamide solutions were collected on a Bruker AM-500 (125.7 MHz) spectrometer with a 5 mm inverse broadband probe using the pulse sequence of Zuiderweg.[19]

Nitrogen-15 NMR spectra of the [1-^{15}N]-labeled nicotinamide salts were obtained at 32 °C in 20 mm tubes at 40.6 MHz on a Bruker AM-400 spectrometer using a 20 mm broadband probe. Data were collected using a spectral width of 9 KHz with a 50° pulse angle and a 2 s relaxation delay. Proton decoupling was not employed in order to avoid signal loss due to a negative nuclear Overhauser effect. Nitrogen-15 chemical shifts are reported as ppm downfield from NH_3, although they were referenced to external $^{15}NH_4Cl$ (23 mM) in 1 M HCl (10% D_2O), which was assigned a shift of 24.93.[20] The chemical shift of the reference was determined both before and after the acquisition of each spectrum.

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