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Carbon-13 Nuclear Magnetic Resonance Studies of Vitamin B_6 Schiff Base and Carbinolamine Formation in Aqueous Solution.[1] 1. The Adduct of Pyridoxal 5′-Phosphate and DL-Alanine

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Abstract: The Schiff base and carbinolamine formation from pyridoxal 5′-phosphate and DL-alanine in aqueous solution was investigated by carbon-13 nuclear magnetic resonance spectroscopy. The pK_a value for the deprotonation of the pyridinium nitrogen was found to be less than that of free pyridoxal 5′-phosphate. At pH 7.1 two pH-dependent forms of the Schiff base and three species of carbinolamines which have different configurations were detected, while at pH 10.5 the pH-dependent forms of the Schiff base predominate. At pH 6.3 the Schiff base is equally distributed between the pH-dependent forms and increased concentration of the three carbinolamine species were detected. Evidence presented suggests that the Schiff bases allow for no intramolecular interactions between the iminium proton and either the phenolate anion of C-3 of pyridoxal 5′-phosphate or the carboxyl group of the amino acid. At pH 12.8, the equilibrium is shifted from the Schiff base toward free components and the carbinolamine intermediate was not clearly detected.

Vitamin B_6 compounds are known to be essential in enzymatic metabolism of amino acids. The early studies by Braunstein et al.[3] and Snell et al.[4] suggested that the initial step in the metabolic mechanism of amino acids is Schiff base formation between the formyl group of pyridoxal 5′-phosphate and the amino group of the amino acid.

Considerable information on the equilibrium of Schiff bases formed by pyridoxal or pyridoxal 5′-phosphate with amino acids or amines has been obtained from UV–visible and ^1H NMR studies in aqueous and nonaqueous media,[5-14] but structural evidence for these dynamic states has not been conclusive. Furthermore, the tetrahedral intermediate (carbinolamine) formed through the addition process between the carbonyl and the amino group of the two components has received little structural attention.

Recent studies of vitamin B_6 and derivatives[15,16] and amino acids[17,18] by carbon-13 nuclear magnetic resonance spectroscopy (^{13}C NMR) have led us to pursue the application of ^{13}C NMR methods for the derivation of dynamic structural information in the formation of Schiff bases and carbinolamine complexes from pyridoxal 5′-phosphate and amino acids.

This study is the first comprehensive treatment of ^{13}C NMR application to Schiff base and carbinolamine formation from pyridoxal 5′-phosphate and amino acids, although a preliminary study on pyridoxal 5′-phosphate–amine systems was reported very recently.[19,20] We wish to establish a correlation of chemical shifts with Schiff base structures and to provide ^{13}C NMR evidence for the intermediacy of carbinolamines.

Experimental Section

Pyridoxal 5′-phosphate was purchased from Sigma Chemical Co. and DL-alanine was obtained from Merck and Co. Reagents were used without further purification. D_2O obtained from Diaprep was 99.7% pure. The NaOD was prepared from D_2O and metallic sodium under dry nitrogen.

^{13}C NMR spectra were obtained at 25 °C on a Bruker HX90E pulse Fourier transform NMR spectrometer (22.63 MHz) interfaced with a Nicolet 1080 computer. Typical parameters for ^{13}C NMR experiments follow: spectral width of 6024 Hz with acquisition of 8 K data points, 7-μs pulse corresponding to a tip angle of 30°, and a recovery time of 2 s. The number of spectral accumulations was in the range of 5000–7500 depending on sample conditions. Chemical shifts are given in parts per million (ppm) downfield from external tetramethylsilane (capillary with 5-mm o.d. concentric tube within the 10-mm sample tube). The digital reproducibility is ±0.1 ppm. The probe temperature was 25 °C. D_2O solvent was the source of an internal deuterium lock. Broad band proton noise decoupling and gated decoupling experiments were carried out by standard methods.

The sample solution was prepared by first dissolving the amino acid in the 0.35 M pyridoxal 5′-phosphate (pH 6.0) stock solution and then adjusting to the final concentration and pH. Sample concentrations were 0.3 M in each component. Before preparing the stock solution,

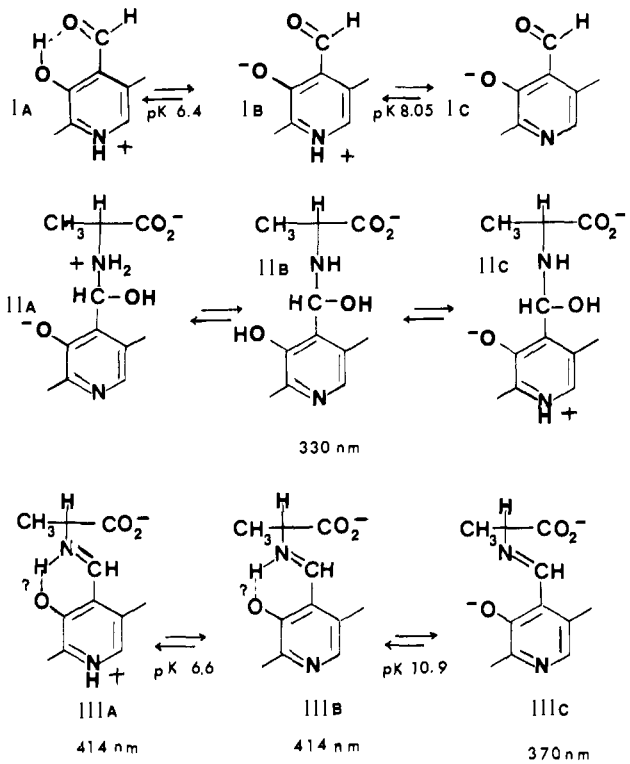

Figure 1. Structural and spectral species present in a mixture of pyridoxal 5′-phosphate and alanine.

D_2O was purged with nitrogen gas. Spectral data were collected within 5 h after sample preparation. The time limitation was included to avoid slower side reactions such as vitamin B_6 catalyzed reactions of amino acids,[20] which were ascertained by ^1H NMR spectroscopy. Acidity was varied by the addition of NaOD. Under these conditions and at these concentrations pH is a working concept and are direct readings of the pH meter without corrections to pD. Therefore, results reported here must be viewed as approximations of pH.

Results and Discussion

UV and visible spectroscopy of aqueous solutions of pyridoxal phosphate and amino acids shows that extensive imine or Schiff base formation occurs over a wide pH range.[5]

In Table I, we have compared the ^{13}C NMR data of the equilibrium states formed with 0.3 M pyridoxal 5′-phosphate and 0.3 M DL-alanine in aqueous solution at three different pHs. The resonances arising from free components were easily assigned in each case from the pH-dependent spectra of pyridoxal 5′-phosphate and DL-alanine.

The spectra at pH 6.3 and 7.1 are more complex than those at pH 10.5 (Figure 1), indicating greater Schiff base formation at the higher pH value. The Schiff bases formed in the neutral pH range are yellow and absorb light maximally at about 400 nm (Figure 1). An obvious indication of Schiff base formation in the NMR case is the decrease in intensity of the formyl carbon (197.1 ± 0.3 ppm) of pyridoxal 5′-phosphate. The Schiff base formed shows two pK values. The first pK of 6.6 is associated with ionization of the pyridine nitrogen and results in formation of the anion IIIB (Figure 1). No UV visible spectral changes are reported or associated with this first dissociation, which seems surprising.[5] The anion form IIIB (Figure 1) has a proton associated with or residing on the imine nitrogen which may or may not be hydrogen bonded to the phenolic oxygen at the 4 position over the pH range 7.1–10.5. The anion IIIB is the major UV-visible spectral species over the pH range 7.1–10.5 and has a λ_{max} of 414 nm.

Our approach to studying the pH dependence on Schiff base formation was to correlate ^{13}C chemical shift data with the structures of various possible species. The intense lines observed

Table I. ^{13}C NMR Chemical Shifts of Pyridoxal 5′-Phosphate–DL-Alanine System in Aqueous Solution

pH		C-2	C-2′	C-3	C-4	C-4′	C-5	C-5′	C-6	C-α	C-β	CO_2
	IA	152.7	17.4	*a*	127.2	197.1	137.5	62.7	125.4	51.7	17.4	177.1
	II	146.6										
7.1		145.9	16–17[c]	163–164[c] (triplets)	125–138[c]	68.7	130–136	61–64[c]	125–137	61–64[c]	16–17[c]	*a*
		145.4										
	IIIB⇄ IIIC	157.1	19.1	168.3	116.9	164.9	133.5 132.8	62.7	133.1	63.5	20.7	178.2
	IB	157.4	*a*	*a*	125.2	*a*	133.6 133.3	*a*	130.6	52.2	*a*	*a*
10.5	I[b]	157.4	20.0	168.1	125.2	197.6	134.0 133.0	63.5	129.3			
	IIIB⇄ IIIC	157.4	19.6	168.1	116.8	165.1	132.6 132.3	62.6	132.3	63.7	20.7	178.5
	IA	152.6	17.0	164.5	127.3	197.1	136–137	61.8	125.2	51.7		
	II	146.5										177–178[c] (doublets)
6.3		146.1	15–18[c]	163–165[c] (triplets)	124–137[c]	68.5						
		145.5					124–137[c]	61–64[c]	124–137[c]	61–64[c]	16–18[c]	
	IIIA	156.3	20.5	168.7	117.7	163.9						

[a] The concentration is not enough to be detected. [b] Resonances from the binary system of aqueous pyridoxal 5′-phosphate solution at pH 10.5. [c] Approximately estimated range; the resonances cannot be distinguished because of the overlapped complexity.

in the spectrum at pH 7.1 became more intense at pH 10.5 while those with low intensity at pH 7.1 diminished further at pH 10.5 (Figure 2a and b) which is indicative of greater Schiff base formation. The reverse situation is true at pH 6.3 (Figure 2c). It is suggested that the lines of high intensity at pH 10.5 are due to the time-averaged chemical shifts of the equilibrium state between imine anion and dianion (IIIB ⇄ IIIC).

In the spectrum taken at pH 10.5 the formyl carbon resonance of pyridoxal 5′-phosphate completely disappeared, which indicates that Schiff base formation is favored at a moderate alkaline range. The dianion which also exists at these pH values is a weak acid with a pK of 10.9.[21] The dianion form has an absorption band at about 370 nm (Figure 1). The increased Schiff base formation is consistent with the increased concentration of the nucleophile (the deprotonated amino acid) under alkaline conditions. Furthermore, under these conditions, base-catalyzed dehydration of carbinolamine is favored (further discussion will be given on this intermediate). The resonances of the equilibrium state between the imine anion (IIIB) and the imine dianion (IIIC) are pH independent in the pH range 7.1–10.5, and the equilibrium is sustained in the mixture even as low as pH 7.1. Assignments of the resonance lines for the equilibrium state of these two Schiff bases (IIIB and IIIC) were made not only on the basis of expected chemical shifts but also from gated decoupling data. Carbons CO_2^-, C-2, C-3, C-4, and C-5 appeared as singlets, C-α, C-4′, and C-6 as doublets, C-5′ as a triplet, and C-β and C-2 as quartets. At lowest field (178.5 ppm) is the carboxyl group and the strong signals at 169.1 and 157.4 ppm must be due to C-3 and C-2, respectively, based on the deshielding of phenolate anion and the pyridine nitrogen. Assignment of C-4 at 116.8 ppm and C-5 at 132.0 ppm was done on the basis of the expected influence on chemical shifts upon Schiff base formation. In the ^{1}H noise-decoupled spectrum, C-5 appeared as a doublet at 132.4 ppm. The observed coupling constant ($^3J_{POCC}$ = 8.0 Hz) is consistent with a trans arrangement about C5′–O bond.[16] Incidental overlap of C-6 (132.3 ppm) and one of the peaks of C-5 was confirmed by gated decoupling and noise-modulated off-resonance decoupling experiments. Assignment of C-4′ at 165.1 ppm (J_{C-H} = 174.3 Hz) is based on the observation of similar values for imine carbons.[20] Appearance of C-α at 63.7 ppm (J_{C-H} = 141.9 Hz) is in the range expected for such a carbon. An unambiguous differentiation between C-β and C-2′ was not possible and the assignments made in Table 1 and Figure 2 for these resonances may be reversed.

As previously stated, the two Schiff bases (111B and 111C) are in equilibrium as the major species at pH 7.1. In Table 1, it was shown that the chemical shift of pyridoxal 5′-phosphate at pH 10.5 is very similar to that of the equilibrium state Schiff base formed at pH 7.1, which suggests that the pK_a of the pyridinium nitrogen of pyridoxal 5′-phosphate has decreased upon Schiff base formation. While we were preparing this article, we were informed that the pK_a is 6.6 as determined by UV-visible spectroscopy.[22] A very recent report[23] on the nature of binding of pyridoxal 5′-phosphate and the corresponding Schiff bases to glycogen phosphorylase lends further support to the above.

On the basis of the chemical shift correlation established for the equilibrium state Schiff base (IIIB ⇄ IIIC) structural assignments of the species observed at pH 6.3 can be made. Evidence for the Schiff base formation under these conditions comes from the observation of the C-4′ resonance at 163.9 ppm. This iminium nitrogen has a pK_a of 10.9.[22] The ^{13}C chemical shift of the C-4′ resonance shows little variation over the pH range of 7.1–10.5, even up to 12.8. This observation would suggest that the iminium proton remains associated over a wide range of pH which may extend up to a pH of 13.9 if we rely on the above value for the pK_a. Unfortunately, the ^{13}C chemical shift of the C-4′ could not be examined at such a high

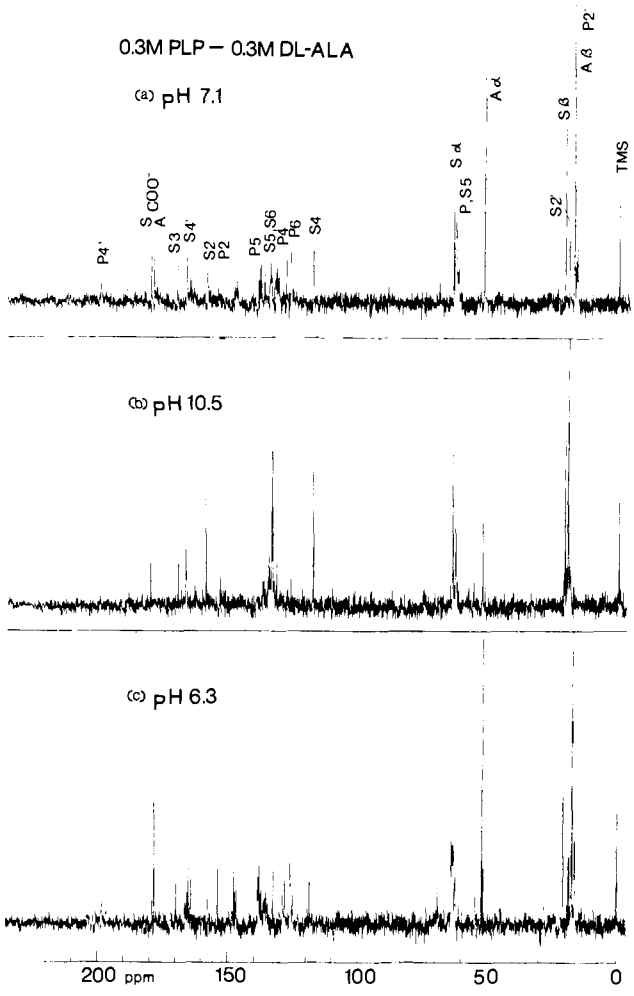

Figure 2. ^{13}C NMR spectra of 0.3 M pyridoxal 5′-phosphate (PLP)–0.3 M DL-alanine (DL-ALA) at three different pHs. P and A: resonances of pyridoxal 5′-phosphate and DL-alanine in free states in the system. S: resonance of dianion Schiff base.

alkaline pH because of the low concentration of Schiff base under these conditions. Consequently, we are led to assume that the resonances observed at pH 10.5 and 7.1 are due to the equilibrium state Schiff base (IIIB ⇄ IIIC).

Observation of the C-2 and C-2′ resonances of the Schiff base at 168.3 and 20.5 ppm, respectively, is a clear indication that the pyridine nitrogen of the Schiff base formed at pH 6.3 is in the deprotonated state as both these resonances would be several parts per million upfield if the pyridine nitrogen were protonated. On the basis of the resonances of C-4′, C-2, C-2′, and C-3 (168.7 ppm) a plausible structure for the species is IIIA. The C-3 resonance at 168.7 ppm shows little change from that detected at pH 10.5 (168.1 ppm) and furthermore at pH 12.8 (168.3) which suggests that the phenolate anion at C-3 in IIIA is not involved in hydrogen bonding to the iminium proton in spite of the extremely high pK_a of this proton which would be explained by hydrogen bonding. The x-ray structure of the corresponding chelated Schiff base shows a metal–oxygen distance of 1.9–2.1 Å,[24,25] a distance too large for the imine proton to assume a hydrogen bond with the phenolate anion, although the crystal structure is not always extrapolable to the corresponding solution structure. As previously mentioned, close examination of the resonances over the range 15–20 ppm where C-2′ resonance occurs provides information on whether the pyridine ring nitrogen is protonated or deprotonated. In the deprotonated form (IIIA), the C-2′ resonance occurs at 20.5 ppm. All of the other peaks in this region at pH 6.3 occur upfield from 18 ppm. One of the peaks in this 15–

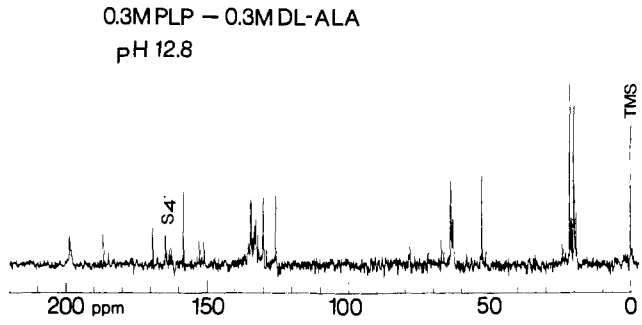

Figure 3. ^{13}C NMR spectrum of 0.3 M PLP-0.3 M DL-ALA at pH 12.8.

18-ppm region is due to C-β of IIIA and the other resonances in this region suggest the presence of species where both the α-amino group and the pyridine nitrogen are protonated. Alanine with a protonated α-amino group has its C-β resonance at 16–17 ppm at pH 6.3.[18] Also as protonation at N-1 of the pyridine ring of vitamin B_6 compounds results in an upfield shift of the C-2′ resonance to 16–17 ppm,[15,16] two of the lines may result from C-β and C-2′ of the free components. The remaining lines, then, should be attributed to some other plausible species, some of whose carbons exhibit triple resonances at 160–163 and also at 145–147 ppm. Those resonances were also detected at pH 7.1 with less enhanced intensity than at pH 6.3. The C-2 resonance at 145–147 ppm and C-3 at 160–165 ppm are common for some vitamin B_6 compounds where C-4′ is in an sp^3-hybridized state such as pyridoxine, pyridoxamine, and pyridoxamine 5′-phosphate.[15,16] This trend is also present in amine adducts of pyridoxal 5′-phosphate where C-4′ is sp^3 hybridized. In addition, the range 145–147 ppm is much further upfield than one would expect for pyridoxal 5′-phosphate and its Schiff base derivatives. The reasons outlined above lead us to suggest that at pH 6.3 and 7.1, tetrahedral intermediates such as three different species of carbinolamine may account for the spectral observations. The closeness of the triplets of C-2 and C-3 observed at 145–150 and 160–165 ppm provides some support for the similarity in the structures.

One might anticipate this observation to be due to the existence of the three possible carbinolamine species which differ by protonation (IIA, IIB, and IIC) as intermediates. Some recent kinetic studies of carbinolamine and imine formation between carboxyl compounds and highly basic amines lend support to the existence of these forms.[26] However, this possibility is not likely to be applicable here since protonation is fast on an NMR time scale and only average chemical shifts can be observed. Another possible explanation for three species of carbinolamine could be configurational forms arising from hydrogen bonding involving the phosphate group and the amino group.

The existence of the carbinolamine (IIC) formed in the condensation process between pyridoxal 5′-phosphate and amines was speculated by Honikel and Madsen[27] although the original proposal for the presence of this species was implied by Kent et al.[28] The resonance at 68.5 (pH 6.3) and 68.7 ppm (pH 7.1) is probably due to the C-4′ of one of the carbinolamine species. Several other observations should be clarified. The resonance of the carboxyl carbon of the pyridoxal 5′-phosphate–alanine complexes does not show much variation in chemical shift in the range pH 10.5–6.3. At pH 10.5, the Schiff base is in the dianion form and no hydrogen bond formation with the imine proton is possible. At pH 6.3, however, the imine moiety of the Schiff base is protonated. From the chemical shift data, it appears therefore that the carboxyl carbon is not involved in intramolecular hydrogen bonding with the imine proton even at pH 6.3. The x-ray results[24,25] provide some support for our conclusion by showing that the chelate Schiff base has a distance of 1.9–2.2 Å from metal to carboxyl group removing the possibility of intramolecular hydrogen bonding. Our results are in contrast to the speculation made by Martell and his co-workers.[11,29] Further, the ^{13}C chemical shift for C-3 in the Schiff bases is almost invariable (within experimental error) over the pH range 6.3–10.5, implying no hydrogen bonding to the iminium proton. Suggestions made previously on the basis of UV-visible[10] and ^1H NMR data[11] are not in agreement with this. Evidence for the keto enamine suggested by early studies of Metzler[5,10] and Heinert and Martell[29] was not clear in these studies. However, the present detection of carbinolamine intermediates makes the possible existence of keto enamine structure somewhat unlikely. Pyridine derivatives which lack a double bond in conjugation with the aromatic ring absorb at wavelengths below 330 nm[22] whereas the free aldehyde form of pyridoxal phosphate and its Schiff bases absorb above 390 nm.[22]

In the Schiff base studies of pyridoxal 5′-phosphate and tris(hydroxymethyl)aminomethane, it was reported that a carbinolamine was in equilibrium with free components.[14] In the ^{13}C NMR spectrum obtained at pH 12.8 (Figure 3), the appearance of the C-4′ resonance at 197.6 ppm is an indication of an equilibrium shift to free pyridoxal 5′-phosphate. The peak at 163.9 ppm due to C-4′ of the Schiff base (IIIB) is still present. This is consistent with our ^1H NMR results[30] which showed that the Schiff base IIIC was in dynamic equilibrium with free components in high pH values. The resonance pairs observed at 150–153 and 160–163 ppm for the C-2 and C-3 carbons, respectively, may arise from two carbinolamines or one carbinolamine and a high pH-dependent Schiff base. However, the concentration of carbinolamines were not sufficient to be detected by ^1H NMR experiments.[30] Observation of six ^{13}C resonances in the CH_3 region (19–22 ppm) is indicative of the presence of multiple species such as free Schiff bases of the C-α deuterated and undeuterated, and carbinolamine. The downfield shifts of the carboxyl resonances to 184–186 ppm at this high pH for both free and Schiff base forms are probably due to the reorganization of the solvation state around this group.

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Crystal Structure of the Ion Pair 1-Methyl-3-carbamidopyridinium *N*-Acetyl-L-tryptophanate, a Model for 1-Substituted Nicotinamide–Protein Charge-Transfer Complexes

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Abstract: The π–π^* charge-transfer complex, 1-methyl-3-carbamidopyridinium *N*-acetyl-L-tryptophanate, a model for charge-transfer complexes between 1-substituted nicotinamide derivatives and proteins, was investigated by the method of x-ray structure analysis. The phase problem was solved by direct methods and the structure was refined by standard methods (final $R = 0.057$). The yellow crystals are elongated in the $\vec{c}$ direction and the ion pairs form an extended alternating donor/acceptor stack in this direction, the planes of a donor/acceptor pair making an angle of 9° with each other. Within the stack, the ring–ring distances between a tryptophan donor and one of its neighbor acceptors are somewhat shorter than to the other neighbor. The donor/acceptor pair with these shorter distances also exhibits a larger degree of mutual ring–ring overlap, suggesting that discrete charge-transfer pairs may be favored within the stack. The hydrogen bond network links a given tryptophan moiety exclusively to the neighbor acceptor with the longer ring–ring distances. This network, combined with the attractive forces derived from the charge-transfer interaction, could explain the crystal habit. The calculated permanent dipole moments of the donor and acceptor are uncoupled in both the ground state and the excited state. The contribution of dipole–dipole interactions to the specificity of binding of NAD^+ to proteins is discussed.

The indole moiety of tryptophan forms a π_D–π_A^* charge-transfer complex with nicotinamide adenine dinucleotide (NAD^+) in model studies[2] and it has been proposed that this kind of complex is responsible for the long-wavelength absorption in NAD^+/3-phosphoglyceraldehyde dehydrogenase mixtures.[3] Similar complexes are formed between 1-substituted 3-carbamidopyridinium ions and various tryptophan derivatives as well as exposed indole groups in several proteins.[2a,4-8] The complexes of proteins with 1-methyl-3-carbamidopyridinium chloride (1-methylnicotinamide chloride) have been used extensively to study the environments of tryptophanyl and tyrosyl residues with regard to their solvent availability as a function of solvent composition.[4-9]

The nature of the surface interaction necessary for the formation of these π–π^* charge-transfer complexes is of some biological interest in that it may in part explain the specificity of binding of the coenzyme NAD^+ to various enzymes and provide a more detailed understanding of how the environment of a tryptophanyl residue affects the binding of nicotinamide analogues in solution. The interaction geometry of 1-(2-indol-3-ylethyl)-3-carbamidopyridinium chloride (I), an intramolecular model for these complexes, has been studied both in solution[10] and by the technique of x-ray crystallography.[11] These experiments indicated that in solution the compound adopted a folded gauche conformation while in the crystal the two ring systems were in an extended trans configuration. However, in the solid state the donor and acceptor moieties from adjacent molecules have a large amount of π-orbital overlap and alternate in an extended donor/acceptor stack in a manner similar to that of previously determined π-donor/π-acceptor complexes.[12] In the solid state, the calculated permanent dipole moments of the donor/acceptor pairs were apparently strongly coupled; this observation was subsequently used as part of a possible criterion for a stereospecific interaction between nicotinamide and tryptophan.

In an attempt to gain further insight into the potential energy minimum occupied by indole/nicotinamide charge-transfer complexes and the possible effects of crystal packing