Effects of hydration on the acid–base interactions and secondary structures of poly-L-lysine probed by ^{15}N and ^{13}C solid state NMR†

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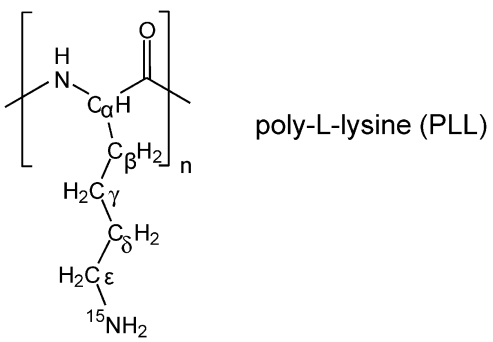
Using high resolution solid state ^{15}N and ^{13}C NMR spectroscopy we have studied the effects of successive hydration on the ^{15}N labeled side chain amino groups of solid poly-L-lysine (PLL) in the presence of acids. Generally, hydration leads to the formation of local "ionic fluid" phases composed by flexible side chain ammonium groups, acid anions and small amounts of water. The associated local dynamics reduces the widths of the inhomogeneously broadened ^{15}N amino signals found for the dry states. The hydration of free base PLL—which consists of mixtures of α-helices and β-pleated sheets—is monitored by a small low-field shift of the amino group signal arising from hydrogen bonding with water, reaching eventually the value of PLL in water at pH 13. No difference for the two conformations is observed. PLL×HF adopts a similar secondary structure with isolated NHF hydrogen bonds; hydration leads only to small low-field shifts which are nevertheless compatible with the formation of ammonium groups in aqueous solution. PLL doped with small amounts of HCl contains ammonium groups which are internally solvated by neighboring free amino groups. Both nitrogen environments are characterized by different chemical shifts. Hydration with less than one water molecule per amino group leads already to a chemical shift averaging arising from fast proton motions along NHN-hydrogen bonds and fast side chain and anion motions.

By contrast, the hydration of fully doped PLL×HBr and PLL×HCl is more complex. These systems exist only in β-pleated sheet conformations forming alkyl ammonium salt structures. Separate ^{15}N signal components are observed for (*i*) the dry states, for (*ii*) wet β-pleated sheets and for (*iii*) wet α-helices which are successively formed upon hydration. Exchange between these environments is slow, but water motions lead to averaged amino group signals within each of the two wet environments. These results indicate that the different environments form domains. As the replacement of NHBr or of NHCl hydrogen bonds by NHO hydrogen bonds leads to high-field shifts the observation of separated signals is the result of different water content in the three domains. In agreement with previous X-ray powder diffraction studies we observe a dominance of the α-helical regions at above 3 water molecules per amino group in the case of PLL×HBr and at about 5 water molecules in the case of PLL×HCl, an effect arising from the limited space between β-pleated sheets and the larger volume of bromide as compared to chloride.

Introduction

Poly-L-lysine (PLL, Scheme 1) is an important poly-amino acid which is used in numerous materials and drug delivery systems. In the past, mainly the secondary structure of PLL in aqueous solution and in the solid state has been the focus of interest, *i.e.* the structure of the main chains, but little is known about the space in between. In aqueous solution, the secondary structures of poly-L-lysine have been studied using various spectroscopic techniques, ranging from optical methods to NMR.[1] At low pH, a random coil conformation is formed. Raman optical activity[2] studies showed that this



Scheme 1 Chemical structure of labeled poly-L-lysine-$^{15}N_{\varepsilon}$ (PLL).

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† Electronic supplementary information (ESI) available: Table S1. ^{15}N chemical shifts of PLL in water as function of pH according to ref. 16. Table S2. ^{15}N chemical shifts and half widths of dry and hydrated samples lyophilized at different pH. Fig. S1. ^{15}N NMR spectra (60.8 MHz) of dry and wet PLL×HCl lyophilized at pH 2. For comparison, the liquid state NMR spectrum of Fig. 4 obtained at pH 4 is included. See DOI: 10.1039/c002730h

structure exhibits regions corresponding to a mixture of α-helices, β-pleated sheets and left-handed helices. At pH 9–11 a transformation to α-helices is observed by ^{13}C NMR.[3] Heating the basic solution leads again to the formation of β-pleated sheets as revealed by ESR and NMR,[4] optical rotatory dispersion measurements[5] and Raman spectroscopy.[6] IR measurements showed that the secondary structure is also influenced by the chain length[7,8] According to Scheraga, the β-pleated sheet structure is favored by hydrophobic interactions between the side chains by which the entropy of the surrounding water is increased.[9] Finally, circular dichroism techniques are able to detect secondary protein conformations which are influenced by water in confined space.[10]

X-ray diffraction studies of PLL×HCl[11] and PLL×HBr fibers[12] containing small amounts of water revealed β-pleated sheet structures in which the amino groups form halide salts. Complete removal of water leads to amorphous structures, exhibiting an NH stretching band at 3030 cm^{-1}.[13] On the other hand, when PLL×HCl and PLL×HBr were successively hydrated, the distances between the β-pleated sheets increased from about 15 Å to 17 Å. It was found that PLL×HCl could accommodate up to 5 and PLL×HBr up to 3 water molecules. Unfortunately, the locations of the water molecules could not be determined by X-ray powder diffraction. It was proposed that they are located in empty pores of the side chain structure. At higher relative humidity hexagonally arranged α-helices were observed. These observations were in line with the finding that wet PLL×H_3PO_4 crystallized as hexagonally arranged α-helices.[14]

For the function of lysine side chains in proteins the protonation state of the side chain amino group is important. Using ^{15}N NMR spectroscopy it has been shown that PLL is characterized in aqueous solution by a single pK_a value of 9.85.[15,16] Deprotonation leads to a ^{15}N high-field shift of 8 ppm[16] which is in agreement with the behavior of other amines.[17] The ^{15}N chemical shifts measured at low and high pH refer to the hydrated alkylammonium and alkylamine side chains, whereas they represent averages at intermediate pH.

However, acid–base interactions in the active site of proteins are different from those in aqueous solution.[18] Therefore, we have been engaged in studies by high-resolution solid state ^{15}N NMR CPMAS spectroscopy (CP = cross polarization, MAS = magic angle spinning) of the interaction of the side-chain amino groups of PLL with added acids in the solid state. Whereas we have studied previously the dry solids lyophilized at low pH[16] and then also at higher pH,[19] we report in this paper the effects of hydration which depend on the added acids and on their concentration. Therefore, in order to understand the new results let us firstly summarize our previous findings.

The acid–base interactions of the amino side chains of PLL were shown to depend on the secondary structures which were characterized by ^{13}C CPMAS measurements according to well-established procedures.[20] We were able to identify the ^{15}N NMR signal of free basic amino groups B (Fig. 1a) which stems both from α-helical domains (B^{α}) and from β-pleated sheets (B^{β}) as shown by ^{13}C CPMAS NMR.[16] Small ^{15}N low field shifts indicated that oxygen acids and HF form in the dry solid state only strongly hydrogen bonded complexes which are typical for the gas phase[21] but no salt structures as illustrated in Fig. 1b. For ionization strong electric fields are required; the field strength needed for this process increases when the acidity of HX is reduced in the series from HBr to HF. An alternative is a close contact of many complexes leading to ammonium salts. It was argued that such a close contact is prevented in the case of dry solid PLL interacting with HF or oxygen acids, whereas salt structures are formed with HCl, HBr and HI.[16] On the other hand, ionization may also occur upon the addition of water molecules. This has been verified recently for the system NH_3×HX×mH_2O, where it

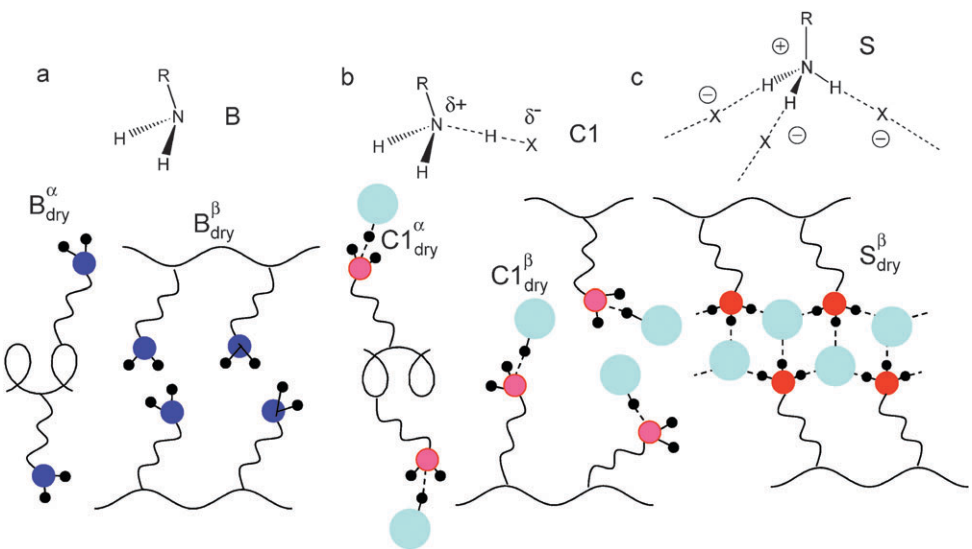


Fig. 1 Hydrogen bonded and protonation states (schematically) of dry solid PLL in the α-helical and the β-pleated sheet conformation according to ref. 16. (a) Acid-free PLL exhibiting free basic amino groups B. (b) Acid–base complexes (C). $C1^{\alpha}$ and $C1^{\beta}$ indicate 1 : 1 complexes in the α-helical and the β-pleated sheet conformation. (c) Salt structures (S).

was found that ionization occurs easily in the case of X = Cl, in contrast to X = F.[22]

In the dry solid state, only the stronger halogen acids HCl, HBr and HI were able to protonate the nitrogen atoms and to form salt structures which we label as "S" according to Fig. 1c. This salt formation was manifested in substantial low-field shifts of the ^{15}N alkylammonium signals. Thus, it was shown that NHX hydrogen bridges can be easily distinguished from NHO hydrogen bridges[16] by ^{15}N CPMAS NMR. Whereas hydrogen bonded complexes of the type C1 could be formed in the α-helical domains ($C1^{\alpha}$) as well as in β-pleated sheets ($C1^{\beta}$) (Fig. 1b), the formation of salt structures was possible only in the β-pleated sheets. This was demonstrated by letting gaseous HCl react with solid free base PLL. In this case, only the β-pleated sheets formed rapidly salt structures whereas the α-helical domains had first to be converted very slowly into β-pleated sheets. This process was catalyzed by water.[19]

Further interesting insights were derived from ^{15}N CPMAS NMR studies of partially acid doped PLL where the number of acid molecules added was smaller than those of the amino groups.[19] Evidence was obtained that in this case PLL can form with HCl not only 1 : 1 complexes $C1^{\alpha}$ and $C1^{\beta}$, but also 2 : 1 and 3 : 1 complexes as illustrated in Fig. 2. Finally, gaseous HCl was let to react with the free amino groups $B^{\alpha/\beta}$ of dry solid PLL lyophilized at high pH. In this case, the free amino groups of the β sheets rapidly formed the salt structure S^{β} whereas the α-helical environments had to be converted firstly to the β-sheets for the reaction to occur. However, a mixture of S^{β} and of B^{α} was not stable, but converted by contact with humid air into $C2^{\alpha/\beta}$ and $C3^{\alpha/\beta}$ mixtures. The different environments did not exchange rapidly and led to inhomogeneously broadened lines.

As we were intrigued by the role of water on the protonation state of the amino acid side chains of PLL and on the conversion of its secondary structures we got involved in the present study which focuses on the events which happen in PLL upon successive hydration. The first goal was to determine using ^{15}N CPMAS NMR whether water which was let to interact with dry PLL×HX, X = Cl, Br breaks the NHX hydrogen bonds, *i.e.* whether it is inserted into the latter or whether it is inserted into empty pores. The second goal was to understand how water molecules induce proton transfer from the acid to the base and whether and how the ions formed dissociate. Finally, we wanted to understand better the increased mobility of the side chains of PLL induced by hydration as observed previously by ^{13}C solid state NMR[23–25] or its combination with incoherent neutron scattering, which has shown a preferential hydration of lysine alkylammonium groups in parvalbumin, a small protein.[25] The question was whether the increased mobility can be observed by ^{15}N solid state NMR and how it is related to proton exchange and acid–base properties.

Experimental

Synthesis

Poly-L-lysine, enriched to about 50% with the ^{15}N isotope in the amino (N_{ε}) side chain positions was synthesized as described previously.[16] The final product PLL×HCl exhibited a molecular weight of 160 000 ± 40 000 Daltons, corresponding to 1000 ± 250 monomers in the polypeptide.

Sample preparation

Free-base PLL or PLL samples doped with the desired acid were obtained by dialysis of PLL×HCl using an anion exchange resin (Bio Rad AGI-X2, 200–400 mesh). The pH of the solutions was then set to the desired value by adding sodium hydroxide solution or the acid studied.

Care had to be taken to avoid contact of the samples to air because the amino groups form carbamic acid in the solid state.[26] Therefore all solutions, the bidistilled water, as well as the anionic exchange resin were degassed in vacuum and were consequently flushed with argon. Finally, the samples were lyophilized.

The lyophilized solids were transferred into rotors and consecutively dried further inside the uncapped rotor at room temperature under vacuum at pressure below 10^{-6} mbar. On average the samples were kept at this pressure for 16–20 h and then flushed with dry argon. The rotors were closed with Teflon sealing caps and measured by NMR.

Hydration of the samples was achieved *via* the gas phase by weighing the filled rotor, removing the cap and storing the uncapped rotor in a dessiccator filled with argon, containing distilled water at the bottom in a separate flask. The samples were kept in this atmosphere between 1 and several days. App. 1 day was necessary in order to achieve an average content of 1 water molecule per amino acid residue. The rotors were then closed, weighed again and measured by NMR. The

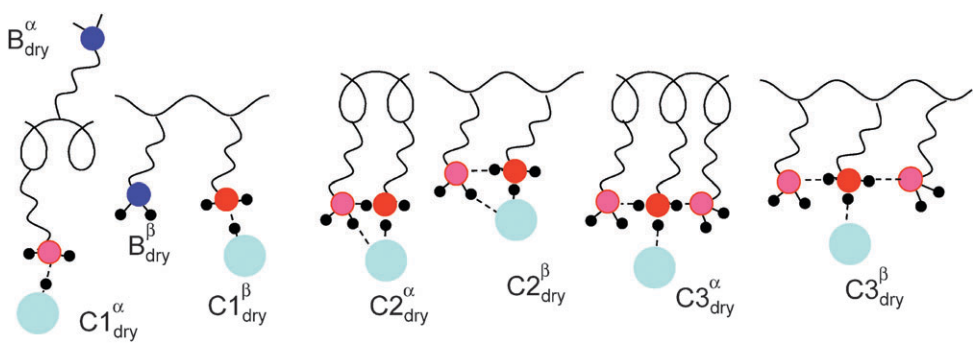


Fig. 2 Hydrogen bonded and protonation states of dry solid PLL at acid fractions $x_{HX} < 1$ according to ref. 19.

amount of water per lysine residue was calculated using the equation

$$n_{H_2O} = \frac{m_{H_2O}}{M_{H_2O}} \Big/ \frac{m_{PLL}}{M_{Lys}} \quad (1)$$

M_{H_2O} is the molecular weight of water (18 g/mol) and $M_{Lys} = M_{HX} + 129.7$ g/mol the effective mass of a lysine residue interacting with the acid HX. For samples lyophilized above pH 10 M_{HX} was set to zero. The total sample mass m_{PLL} was obtained as the weight difference between the sample in the rotor and the empty rotor. The mass of added water, m_{H_2O}, was calculated from the difference of the weight of the "*dry*" sample including the rotor and the weight of the sample after hydration. Naturally, n_{H_2O} represents an average value in the sense that the water distribution between the individual side chains might be inhomogeneous.

We further note that during the lyophilization and drying process acids in excess such as HF or HCl might be partially removed, which can lead to pH changes when the solid is dissolved again in water. For some of these aspects the reader is referred to our previous work.[19] However, note that the pH/pK_a concept is valid only for aqueous solutions, but not for dry or partially hydrated solids. Therefore, no buffer was added to the samples in order to maintain a fictive pH. Moreover, the buffer components would interact directly with the ammonium side chains and mask the interaction with the added acids.

NMR spectroscopy

^{15}N and ^{13}C solid-state measurements were carried out on a Varian Infinity Plus 600 spectrometer at a ^{15}N resonance frequency of 60.8 and a ^{13}C resonance frequency of 150.88 MHz. All measurements were performed at ambient temperature. The ^{15}N solid state NMR spectra were calibrated to external solid NH_4Cl. The ^{13}C NMR spectra were calibrated to solid TSP (sodium salt of 3-(trimethylsilyl)-propionic acid-d_4). Spinning speeds between 5 and 10 kHz were used.

We note that cross polarization from ^{1}H in order to enhance the ^{15}N sensitivity relies on the dipolar interactions. The latter are reduced by anisotropic reorientation and eventually averaged by isotropic reorientation, by which the performance of CP is reduced. Therefore, conventional single pulse ^{15}N MAS experiments were mostly performed to study the hydration of lysine side chains,[23–25] as well as in our ^{15}N hydration studies, using recycle delays of 3 s. We could use this method as the longitudinal relaxation times were of the order of 100 ms to 500 ms, a phenomenon which we ascribe not only to side chain motions but also to NH_3-reorientation, which is preserved in the dry solid. Nevertheless, we could not detect essential differences between single pulse and CP experiments. This might arise from the circumstance that reorientation induced by water is anisotropic, which does not entirely average the dipolar interactions.

The ^{15}N NMR spectra of the aqueous solutions of PLL included in some of the Figs. for comparison were measured previously[16] using a Bruker AMX 500 spectrometer (500.13 MHz for ^{1}H, 50.68 MHz for ^{15}N). Standard inverse ^{1}H decoupled ^{15}N NMR spectra were recorded with a recycle time of 3 s. The spectra were referenced to external solid $^{15}NH_4Cl$, which resonates at −341.168 ppm with respect to external liquid nitromethane.[27,28]

Results

In the following, we report the results of the solid state ^{15}N NMR experiments on samples of PLL partially and fully doped with different acids as a function of the hydration level. The different levels were achieved by exposure for different times to humid argon gas and subsequent weighing of the samples. The spectra were obtained under MAS conditions and ^{1}H decoupling, using CP for dry and 90° pulses for wet samples. For reference also the liquid state ^{15}N NMR spectra obtained under static conditions[16] are included.

Solid state ^{15}N MAS NMR hydration studies of the free amino groups of undoped PLL

The ^{15}N spectra depicted in Fig. 3a show the signal $B^{\alpha/\beta}_{dry}$ of the free amino groups in the dry solid state at −16.5 ppm. Upon partial hydration the signal sharpens and shifts to −14 ppm which is already close to the value of −13.5 ppm for the fully hydrated amino groups in water at high pH.

Solid state ^{15}N MAS NMR hydration studies of PLL×HF

In Fig. 3b are depicted ^{15}N NMR spectra of PLL×HF at various hydration levels. In the dry state, a broad signal is observed at −8.5 ppm. Upon addition of water the signal sharpens and is shifted by about 1 ppm to low field. A signal sharpening occurs already at very low hydration levels. The value of −6.1 ppm for aqueous solution at pH 4 is, however, not yet reached even in the presence of five additional water molecules.

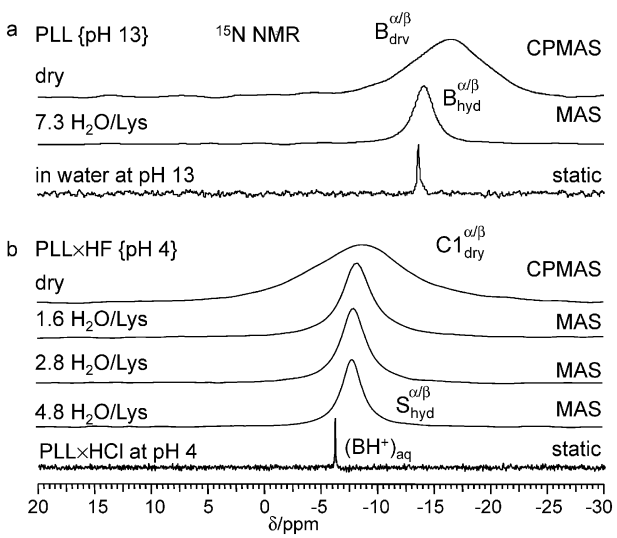


Fig. 3 (a) ^{15}N NMR spectra (60.8 MHz) of dry and wet PLL lyophilized at pH 13. For comparison, the liquid state NMR spectrum of PLL obtained previously[16] at pH 13 is included. (b) ^{15}N NMR spectra (60.8 MHz) of dry and wet PLL×HF lyophilized at pH 4. For comparison, the liquid state NMR spectrum of PLL obtained previously[16] at pH 4 is included.

Solid state ^{15}N MAS NMR hydration studies of PLL×HBr and PLL×HCl lyophilized at low pH

In contrast to solid PLL doped with HF, large spectral changes are manifested upon hydration in the ^{15}N spectra of PLL×HBr and of PLL×HCl, lyophilized at pH 4 as illustrated in Fig. 4 and 5. The low-field components of both amorphous dry solids disappear already at small hydration levels, and two sharper lines appear at higher field. Both lines shift to higher field when the hydration level is increased, and, eventually, a value of about −6 ppm is reached for aqueous solution. Similar spectra are obtained by lyophilization at pH 2. For PLL×HCl, a corresponding signal set is included in the Electronic Supplementary Information. We will discuss the signal assignment and the information arising from these spectra in the Discussion Section.

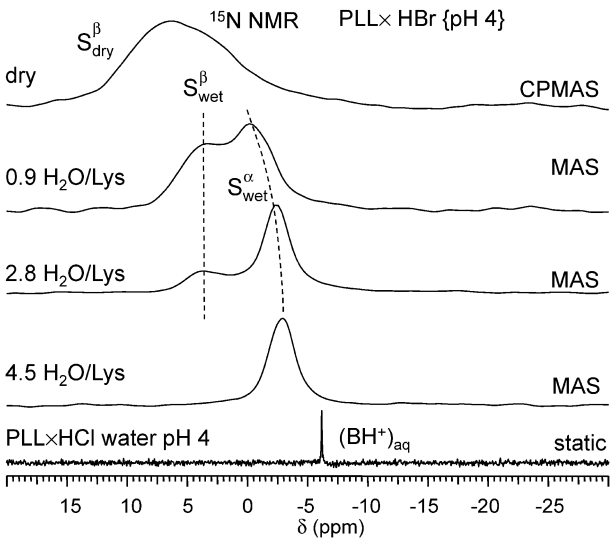


Fig. 4 ^{15}N NMR spectra (60.8 MHz) of dry and wet PLL×HBr lyophilized at pH 4. For comparison, the liquid state NMR spectrum of PLL obtained previously[16] at pH 4 is included.

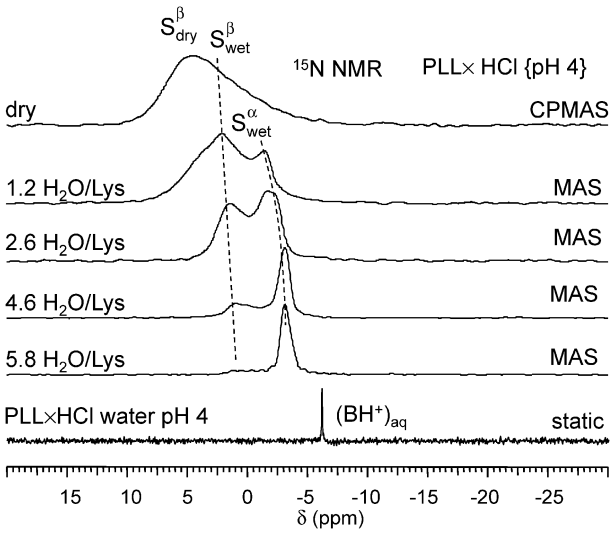


Fig. 5 ^{15}N NMR spectra (60.8 MHz) of dry and wet PLL×HCl lyophilized at pH 4. For comparison, the liquid state NMR spectrum of PLL obtained previously[16] at pH 4 is included.

Solid state ^{15}N MAS NMR hydration studies of PLL×HCl lyophilized at pH > 10

In order to elucidate the response arising from the reduced acid fraction in the samples upon hydration we have performed hydration experiments of PLL lyophilized at pH 10.3 and 10.6. In Fig. 6 are depicted the ^{15}N NMR spectra of a sample of PLL×HCl lyophilized at pH 10.3. According to the pH titration reported previously,[19] the fraction of protonated amino groups at pH 10.3 is about 0.3. A very large inhomogeneously broadened line is observed. Most interesting is that only a very small amount of water is sufficient for a collapse of the broad signal into two relatively sharp lines. For a coalescence of both signals about 3 water molecules per amino acid residue are needed.

In the pH region between 10 and 11 the spectra are subject to drastic changes. At pH 10.6 we expect a fraction of protonated amino groups of about 0.15.[19] Lyophilization at this pH produces a sample whose ^{15}N spectra obtained under MAS conditions and ^{1}H decoupling using 90° pulses are depicted in Fig. 7. After drying in *vacuo* again two signal components are observed, where the low-field component is larger than the high-field component. The latter stems from the free dry amino groups labeled as $B^{\alpha/\beta}_{dry}$ in both the β-pleated sheets and in the α-helices. The broad low-field component arises from the interaction with residual HCl. Upon hydration, both components sharpen and shift towards each other. However, coalescence into a single signal typical for PLL at pH 10.6 is achieved above 5 water molecules per residue. Again, we will discuss the signal assignment and the information arising from these spectra in the Discussion Section.

Solid state ^{13}C MAS NMR hydration studies of PLL×HCl

In the case of PLL×HCl we have performed some additional ^{13}C NMR experiments in order to check for secondary structures and side chain mobilities. In Fig. 8 are depicted ^{13}C CPMAS NMR spectra of dry and wet PLL doped with different amounts of HCl. The assignment of the signals

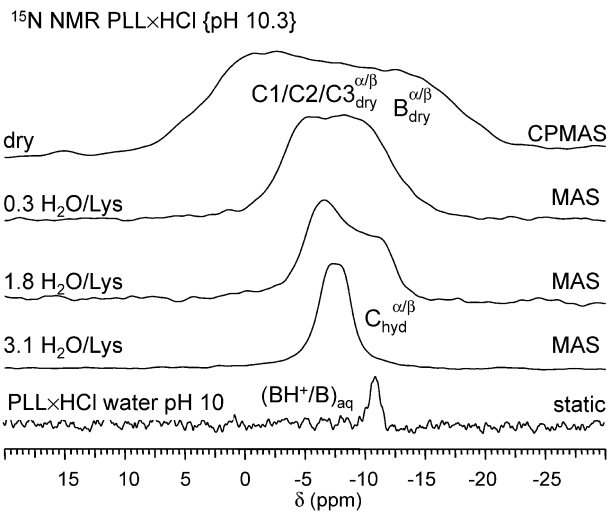


Fig. 6 ^{15}N NMR spectra (60.8 MHz) of dry and wet PLL×HCl lyophilized at pH 10.3. For comparison, the liquid state NMR spectrum of obtained previously[16] at pH 10 is included.

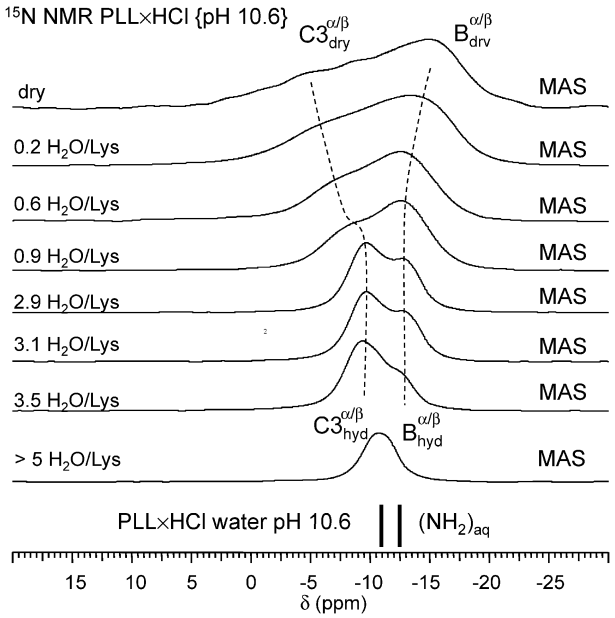


Fig. 7 ^{15}N NMR spectra (60.8 MHz) of dry and wet PLL×HCl lyophilized at pH 10.6. For comparison, the position of the liquid state signal of PLL obtained previously[16] at pH 10.6 is included.

follows the one given previously.[29] In the sample lyophilized at pH 10.3 we observe two lines for the amide carbonyl group at 176 and 172 ppm and two lines at 58 and 52 ppm for the CH carbon of the main chain. This signal doubling has been assigned by Kricheldorf *et al.*[20] Those at 176 and 58 ppm correspond to α-helices and those at 172 and 52 ppm to β-pleated sheets as depicted schematically in Fig. 1. The CO signals exhibit strong rotational side bands indicating a rigid main chain. When 1.4 water molecules per amino acid residue are incorporated the high-field signals sharpen, but the side bands of the CO signals persisted, *i.e.* also the rigidity of the main chain CO groups.

Lyophilization at pH 2 gives rise to broad signals. However, upon hydration the CO signal and the main chain CH signal

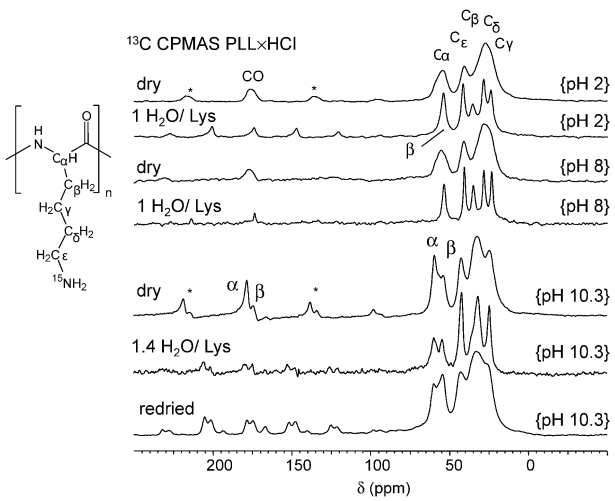


Fig. 8 ^{13}C CPMAS spectra of PLL×HCl lyophilized at pH 2, 8 and 10.3 in the dry and wet state. The side band signals are marked by asterisks.

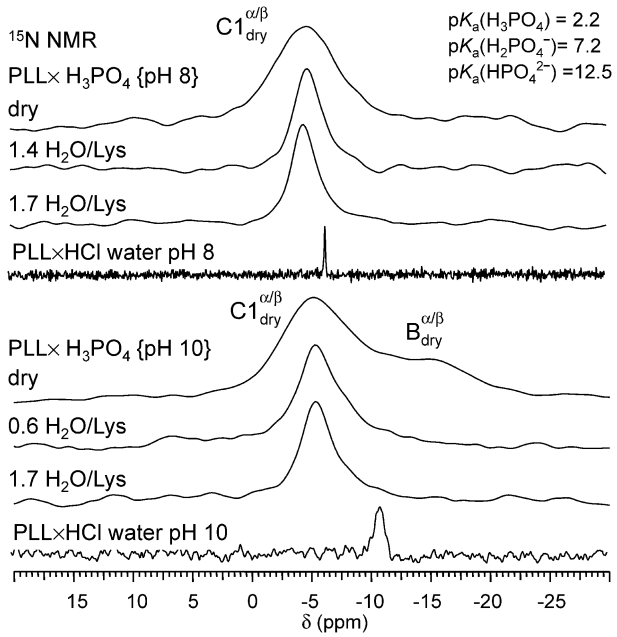


Fig. 9 ^{15}N NMR spectra (60.8 MHz) of dry and wet PLL×H_3PO_4 lyophilized at pH 8 and 10. For comparison, the liquid state NMR spectrum obtained[19] at pH 10.0 is included. The pK_a values stem also from ref. 19.

sharpen and adopt positions typical for β-pleated sheets.[20] However, again the side bands of the CO group remain.

When lyophilization is performed at pH 8 similar results are obtained as at pH 2. This means that at both pH values one acid molecule per amino group is incorporated in the solids produced. This also means that an excess of HCl would be removed *in vacuo*.

Solid state ^{15}N MAS NMR hydration studies of PLL doped phosphoric acids

As an example of a polyvalent oxygen acid we studied the hydration of PLL doped with H_3PO_4. Two samples were studied, lyophilized at pH 8 and 10. In this pH range phosphoric acid is doubly ionized in water,[30] *i.e.* the dominant species is hydrogen phosphate HPO_4^{2-}. The ^{15}N NMR spectra of the dry and wet states are depicted in Fig. 9. They will be discussed later. Again, for comparison, the spectra for aqueous solutions at pH 8 and 10 in the presence of chloride are included.

Discussion

In this section, we discuss the results of the hydration studies of poly-L-lysine interacting with different acids described above, and discuss hydration in terms of a transition from a more or less rigid side chain structure to a kind of local ionic liquid.

^{15}N signal assignments of partially hydrated Poly-L-lysine samples

Free base PLL and PLL×HF. As shown in Fig. 3a, the hydration of the free amino groups shifts the ^{15}N signal from −16.5 ppm to −14 ppm. According to our previous chemical

shift calculations,[16] this shift is mainly influenced by the formation of a hydrogen bond of water to the amino groups as illustrated in Fig. 3a. The value agrees well with the value for aqueous solution at pH 13.

The interaction of free PLL with HF is somewhat stronger than with water and leads to a low-field shift to −8.5 ppm as illustrated in Fig. 3b. This shift has been explained with the formation of hydrogen bonded 1 : 1 acid–base complexes C1 (Fig. 1) in dry PLL×HF, where H is located near the hydrogen bond center, but somewhat closer to F than to N.[16] Such a complex is typical for ammonia–acid complexes in the gas phase.[21] As HF constitutes a weak acid, strong external electric fields are required to shift the proton towards nitrogen. For aqueous solution at pH 4 we expect that HF (pK_a 3.1) entirely protonates the amino groups of PLL which exhibit a pK_a value of 9.85.[16] On the other hand, the ^{15}N chemical shift value of the protonated amino groups in water is −6.1 ppm, *i.e.* not much different from the value of the C1 complex (Fig. 1) of PLL with HF. Thus, in the series of spectra of Fig. 3b proton transfer from fluorine to nitrogen will probably take place upon hydration, in spite of the small chemical shift changes observed. Unfortunately, we cannot decide at which hydration level the dissociation of the hydrogen bonded complex C1 to the hydrated salt may occur. This result supports our recent quantum-mechanical calculations[16] of methylamine–acid complexes where we found out that by chance the ^{15}N chemical shielding of the C1 complex—where H is somewhat closer to F than to N—is the same as for hydrated ammonium. Thus, the absence of a ^{15}N hydration shift for PLL×HF corroborates our spectral assignments.

At this point, we note that DeKock *et al.*[22] have recently performed theoretical studies on the systems NH_3×HX, X = F, Cl hydrated with a different number of water molecules. The cluster NH_3×HCl×H_2O exhibited a strong N···H···Cl hydrogen bond, but ionization took place in NH_3×HCl×$2H_2O$ which behaved as an ion-pair NH_4Cl×$2H_2O$. By contrast, NH_3×HF×H_2O did not form an ion-pair NH_4F×H_2O, nor did a second added water molecule facilitate ionization. These results are in agreement with our interpretation of the effects of hydration on PLL×HF.

PLL×HBr and PLL×HCl. By contrast, PLL×HBr and PLL×HCl behaved in a very different way as illustrated by their ^{15}N spectra in Fig. 4 and 5. The observed spectral features can be assigned with the help of previous X-ray diffraction studies of PLL×HCl[11] and of PLL×HBr[12] at different degrees of hydration. The main results of the latter study are summarized in Table 1. The dry solids are generally amorphous, but very small amounts of water give rise to reflections indicating ordered microcrystalline environments. Two kinds of ordered environments were observed,[11,12] assigned to β-pleated sheets which dominate at lower degrees of hydration and to α-helical structures which dominate at higher degrees. The distance *d* between the β-pleated sheets increased slightly with an increasing number of water molecules per amino acid residue. In the case of PLL×HBr the α-helical structure becomes dominant at about 3 water molecules per residue, whereas 5 molecules per residue are required for this transition to occur in the case of PLL×HCl. This finding was explained with the limited space for water molecules in the β-pleated sheet salt structures and the smaller size of chloride as compared to bromide. However, the locations of the water molecules could not be determined. When large amounts of water molecules are added isotropic solutions are obtained where PLL is assumed to adopt a random coil structure.

Table 1 Summary of X-ray powder diffraction studies of PLL×HCl and of PLL×HBr

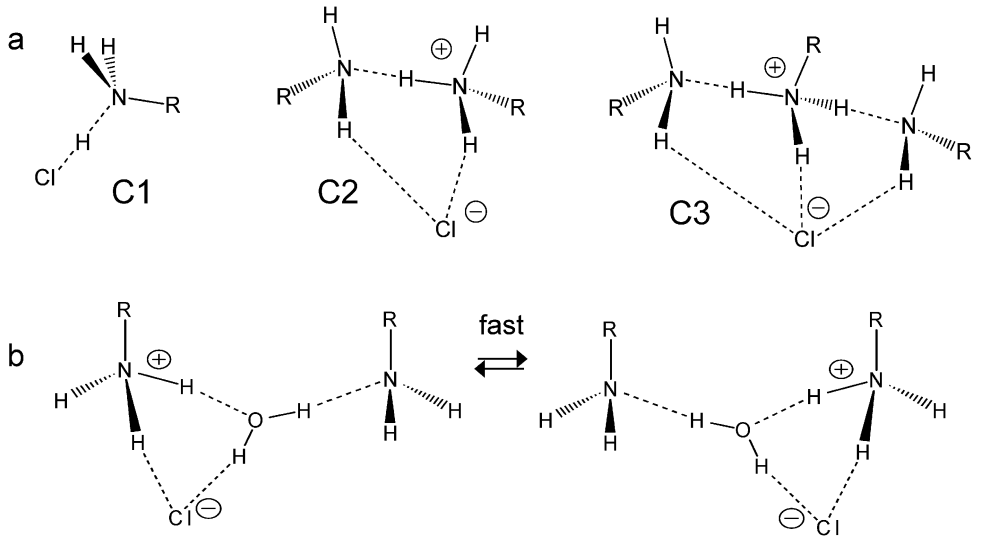
PLL×HCl (parallel chains)[11]			PLL×HBr (antiparallel chains)[12]		
Water/residue	Dominant secondary structure	*d*/Å	Water/residue	Dominant secondary structure	*d*/Å
0	β	15.2	0	β	Amorphous
0.8	β	15.7	0.8	β	16.1
1.4	β	16.2	1.4	β	16.6
1.9	β	16.7	1.9	β	16.6
5	α	16.94	3.2	α	16.8
			5	α	
≈15	Coil (isotropic solution)		≈20	Coil (isotropic solution)	

d: distance between adjacent β-sheets.

Using this information the spectra of Fig. 4 and 5 could be assigned. The signals labeled as S^{β}_{dry} were associated to protonated ammonium groups of dry PLL×HCl and PLL×HCl; they are particularly broad because of an inhomogeneous distribution of chemicals shifts. This finding can easily be explained in terms of non-ideal salt structures. The other signals of Fig. 4 and 5 are substantially sharper in view of the higher degree of crystallinity as well as because of local motions of the side chains due to hydration.[23,24]

Already the addition of small amounts of water leads to a disappearance of S^{β}_{dry} and to the appearance of new lines assigned to S^{β}_{wet}, which shifts somewhat to higher field as the degree of hydration increases. The intensity of this signal decreases, however, upon hydration, and a new signal appears, S^{α}_{wet}, assigned to wet α-helices. This signal further shifts to higher field as the water content is increased. It becomes dominant at about 3 water molecules per residue in the case of PLL×HBr but at about 5 water molecules per residue in the case of PLL×HCl. This finding is in agreement with the X-ray diffraction results and confirms the signal assignments. However, 15 water molecules per residue are needed in the case of PLL×HCl and 20 in the case of PLL×HBr in order to suppress all long-range order and to achieve a situation typical for the aqueous solution.[11,12] This explains that the ^{15}N chemical shift values of −6 ppm at low pH in water are not yet reached in the wet solid state exhibiting 5 to 6 water molecules per residue.

PLL×HCl lyophilized at pH > 10. We come now to the assignment of the ^{15}N NMR spectra of PLL×HCl lyophilized at pH 10.3 and 10.6 which were depicted in Fig. 6 and 7. Small changes of the pH of lyophilization lead to drastical spectral changes. For dry PLL lyophilized at pH 10.3 a very large inhomogeneously broadened line is observed. The high-field component corresponds to dry free amino groups $B^{\alpha/\beta}_{dry}$; according to our previous analysis[19] we assign the lower field



Scheme 2 (a) Hydrogen bonded acid–base complexes (C) of poly-L-lysine with small amounts of HCl. (b) Scenario of hydration assisted proton transfer.

components to a mixture of hydrogen bonded complexes $C_{dry}^{\alpha/\beta} = \{C1_{dry}^{\alpha/\beta}, C2_{dry}^{\alpha/\beta}, C3_{dry}^{\alpha/\beta}\}$ whose structures are illustrated in Scheme 2a. Probably, only the 2 : 1 and 3 : 1 complexes are present.

Most interesting is that only a very small amount of water is sufficient for a collapse of the high and of the low field components into two relatively sharp lines which move towards each other. Both lines must arise from different domains containing different amounts of acid. Within each domain the different environments interchange rapidly because of molecular motions assisted by water molecules. This exchange consists of very complex processes including proton transfer as illustrated in Scheme 2b, hydrogen bond switches and motions of the amino groups, the counterion and water. However, for a coalescence of both signals about 3 water molecules per amino acid residue are needed. Previously,[19] we have obtained evidence that water also catalyzes the interconversion of α-helices and β-pleated sheets. Therefore, it could be that the two line components stem from the two secondary structures arranged in different domains.

Similar effects are observed for PLL lyophilized at pH 10.6 (Fig. 7). Now, the signal $B_{dry}^{\alpha/\beta}$ arising from free amino groups has increased, but a low-field component which we assign mainly to 3 : 1 complexes remains. Upon hydration, the two signal components shift again towards each other, but they coalesce again only at higher water contents.

PLL×H_3PO_4

This sample was studied as an example of the hydration of PLL interacting with a strong oxygen acid, and its model character for protein–DNA interactions. The ^{15}N spectra were depicted in Fig. 9. The pK_a values of H_3PO_4 are 2.2, 7.2 and 12.5.[30] Thus, when PLL is lyophilized with phosphoric acid at pH 8, according to its pK_a values it is plausible that the dominant acid species interacting with the amino groups is HPO_4^{2-}. In the dry solid state a signal is observed at −5 ppm which we assign to a C1 complex. Upon hydration, as in the case of HF, only a small shift is observed for full hydration. However, when the lyophilization takes place at pH 10, in addition to the peak at −5 ppm an additional broad high-field component is observed which arises from free PLL. Upon hydration, the peak at −5 ppm sharpens and the high-field component disappears. However, in water at pH 10 the signal appears at −11 ppm. This signal position is in good agreement with the average signal position of the dry state. In other words, in aqueous solution proton exchange between protonated and free amino groups is fast, but slow in the dry solid state. The disappearence of the high-field signal upon hydration instead of the expected coalescence indicates that not only proton exchange determines the NMR line shapes but also very complex molecular motions induced by the interaction with water.

Mechanisms of hydration of poly-L-lysine and associated phenomena: secondary structure change, heterogeneous *vs.* homogeneous acid distribution, molecular motions, proton transfer, acid dissociation

In this section we discuss scenarios of the changes in acid doped solid dry PLL induced by hydration. Firstly, we discuss in Fig. 10 the case of the free amino groups B and of acids which form hydrogen bonded complexes C1 to C3. Hydration consists of NHO hydrogen bond formation to water molecules which replace the NHX and NHN hydrogen bonds of the complexes. In the case of C2 and C3 complexes the ammonium group structures are preformed in the dry solid state, where the acid proton will be closer to nitrogen than to the acid residue. However, the situation can be different for complexes of the type C1. Such complexes are formed by acids such as HF and oxygen acids at acid concentrations where all amino groups find an acid partner molecule, but where the interactions between complexes are too weak for salt formation. The latter requires stronger electrical dipole–dipole interactions between the complexes.[16] C1 complexes are also formed by HCl and HBr at smaller acid fractions where the distances between the complexes are larger than in the fully doped state.[19] Whereas in complexes of the type C1 the proton is located somewhere in

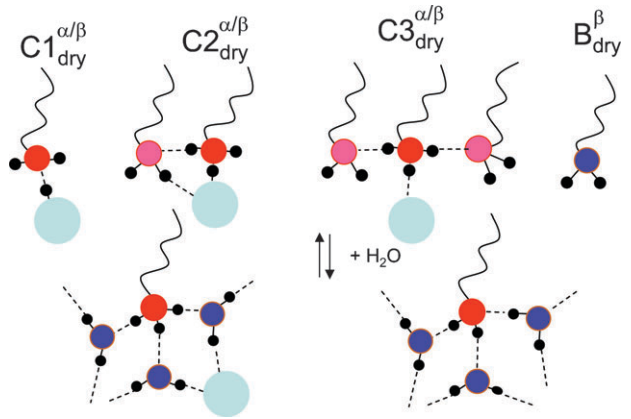


Fig. 10 Scenario of the successive hydration of PLL×HX, X = Cl, Br initially present as salt in the β-pleated sheet conformation. Water is inserted into the NHX hydrogen bonds which increases the distance between the sheets. At large degrees of hydration the α-helical conformation is formed which can adopt more water.

the hydrogen bond, it has been transferred to nitrogen in the hydrated state as long as pH is smaller than the pK_a of 9.85. It is difficult to know exactly how many water molecules are needed for this transformation to occur. The reason is that the ^{15}N chemical shifts of fully hydrated ammonium and of C1 complexes are very similar.[16]

Interestingly, we did not find evidence for a different behavior for β-pleated and α-helical conformations in the case of B and of C1 to C3. However, as illustrated for example in Fig. 8 and 9, we observe different domains, containing different amounts of acids, *e.g.* B and C1 to C3. The addition of a very small amount of water leads to a sharpening of the ^{15}N signals in each domain in which proton transfer processes according to Scheme 2b will then take place. Addition of further water leads to an increased mobility of the amino groups, but a larger amount of water molecules is needed for proton transfer to become fast between the different domains as illustrated. This means that the addition of water produces a kind of local reaction medium in which chemical processes can occur, although PLL still constitutes a solid.

The hydration of the local salt structures of PLL×HCl and of PLL×HBr is different. A scenario arising from the signal assignments proposed in the previous section is presented in Fig. 10. Only the β-pleated sheet conformations can form local salts S in the dry solid state but not α-helical conformations. For salt formation to occur, the latter first have to be converted slowly into the former. This process is catalyzed by water.[19]

The previous X-ray diffraction studies[11,12] indicated that the addition of water leads to an increase of the distance between the β-pleated sheets as indicated in Table 1; however, the locations of the water molecules could not be determined. However, the ^{15}N NMR hydration high-field shifts between NHX in the dry salts and NHO hydrogen bonds of the hydrated ammonium groups are 10 ppm for HCl and even more for HBr and HI as acids.[16] This allowed us to prove that water successively is inserted into the salt structures as illustrated in Fig. 11. Moreover, as β-pleated sheets can incorporate only about 3 water molecules in the case of HBr and 5 in the case of HCl, a larger amount of water molecules induces α-helical conformations, in agreement with the X-ray diffraction studies.[11,12] *Interestingly, proton transfer and hydrogen bond exchange are fast within each secondary structure, but slow between the two structures, which can be explained with the existence of different domains for both conformations.*

The hydration of complexes of PLL with oxygen acids, *e.g.* carboxylic acid, nitric acid, sulfuric acid could not be studied here besides some preliminary experiments on phosphoric acid. In these cases, the anions do not have a simple spherical shape and hydrogen bonds are formed only along given directions. According to previous calculations, the C1 complex

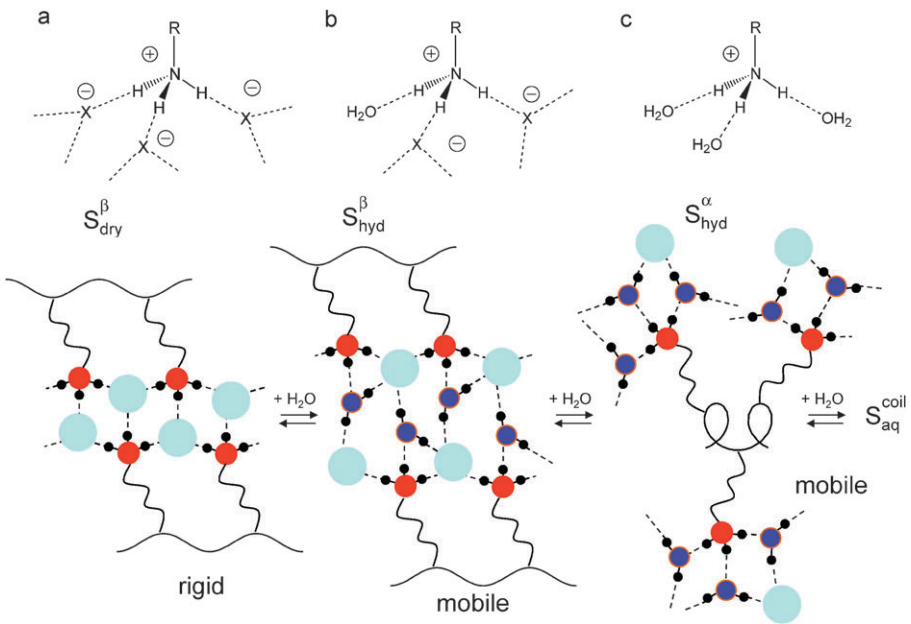
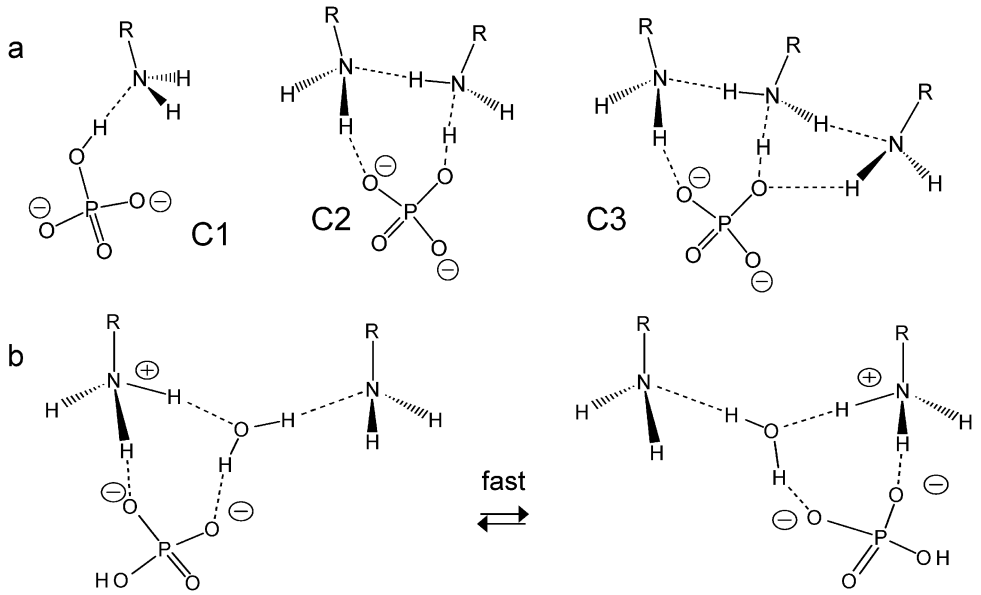


Fig. 11 Scenario of the hydration of acid–base complexes of solid PLL. This mechanism applies for acids such as HF, and also for HCl at small acid concentrations or for free basic PLL, both in the β-pleated sheet and the α-helical conformations.



Scheme 3 (a) Hydrogen bonded acid–base complexes (C) of poly-L-lysine with hydrogen phosphate. (b) Scenario of assisted proton transfer.

of PLL with HPO_4^{2-} exhibits a structure as illustrated in Scheme 3a, where H is located closer to O than to N.[16] The proposed structures of C2 and C3 are tentative. The hydration experiments indicate a similar situation as found for HF. A small number of water molecules leads again to fast proton transfer between free base and complexed amino groups. However, further studies are necessary to understand the hydration of PLL complexed to oxygen acids.

Finally, let us discuss the origin of the increased side mobility of the lysine side chain amino-groups, which has been studied previously by ^{13}C NMR and other techniques.[23–25] The scenarios of Fig. 10 and 11 offer a simple explanation. Without water, the free motion of the amino or ammonium groups is blocked by the interaction with the acids or acid residues. Keeping the main chain and the amino/ammonium groups fixed, the CH_2 groups of the side chain can move fast but can only adopt a reduced number of conformational states. By contrast, hydration destroys the direct acid–base interactions and the ammonium groups as well as the acid anions can adopt many different locations. The mobility induced by hydration may, therefore, not be of a dynamic origin but of the wider accessible conformational space.

Conclusions

We arrive at the following conclusions of this study which will help to understand the ^{15}N NMR properties of amino groups in general as well as the behavior of lysine side chain amino groups in proteins.

(1) *^{15}N NMR hydration and deprotonation shifts*. One needs to distinguish an increase of ^{15}N chemical shielding of protonated amino groups arising from hydration (hydration shift) and arising from the removal of the proton (deprotonation shift). Between the dry solid ammonium salts and ammonium groups in water a hydration high-field shift occurs simply by replacement of the NHX by NHO hydrogen bonds. These shifts are about −15 ppm for iodide, −13 ppm for bromide and −10 ppm for chloride, keeping the NH distances of the ammonium groups more or less the same. Deprotonation of the hydrated ammonium groups in water leads to a high-field shift of −8 ppm.[16] Further dehydration, *i.e.* removal of water, leads only to a high field shift of −2 ppm. The total deprotonation shift in the solid state is then −20 ppm for chloride.

(2) *Hydration induced changes*: Hydration in all samples is not homogeneous and different domains appear. Thus, certain parts are already hydrated, and, therefore, more inclined to changes of the secondary structure. Exchange between domains is slow in the NMR timescale. Therefore, the rate limiting step is slow interconversion of secondary structures between β-pleated sheets and α-helices. In a similar way samples with excess of amino groups exhibit slow exchange between acid containing domains and acid-free domains. However, acid-containing domains consisting of C2 and C3 are supported here: only small amounts of water induce local mobility, and a coalescence of inhomogeneously broadened ^{15}N lines assigned to C2 and C3.

(3) *Hydration induced molecular mobility leading to a local "ionic fluid"*: In the dry solid state the amino groups are fixed by acid–base interactions. Hence the aliphatic side chains exhibit a reduced mobility. However, as has been observed previously using other NMR methods, hydration leads to an enhanced side chain mobility although the samples remain solid.[23–25] Here, we have shown that hydration water destroys the direct acid–base contacts which liberates the side chains, and explains the increase of the side chain mobility. As ions are formed by the water addition, the result is an "ionic fluid" between the more rigid peptide backbone. Nevertheless, secondary structure changes may occur when the hydration level is increased, *e.g.* β-pleated sheets may be converted to α-helices which can accommodate more water molecules to end up eventually in the isotropic liquid. The ionic fluids generated by hydration of solid acid-doped poly-L-lysine may be of special interest as it may be a model environment for the active sites of proteins.

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