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Nitrogen chemical structure in DNA and related molecules by X-ray absorption spectroscopy

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The electronic environment of nitrogen in nucleic acid bases, nucleotides, polynucleotides and DNA has been studied, for the first time using X-Ray Absorption Near-Edge Spectroscopy (XANES). Generally, the spectra of these complex molecules consist of low energy bands corresponding to $1s \rightarrow \pi^*$ transitions and high energy bands corresponding to $1s \rightarrow \sigma^*$ transition, as illustrated using several nitrogen model compounds. The $1s \rightarrow \pi^*$ transitions show particular sensitivity to the chemical environment of the nitrogen. Oxygen substitution on ring carbon atoms generally results in a significant blue shift of the lowest $1s \rightarrow \pi^*$ bands while halogen substitution results in a small blue shift. These observations illustrate the significance of the disturbance of the aromatic ring system produced by exocyclic carbonyl groups. Direct substitution on the nitrogen frequently results in significant spectral perturbations. Differences between the spectra of the polynucleotides and the sums of spectra of the individual nucleotides point to the effects of hydrogen-bonding in complementary double-helix structures. The XANES spectrum of a DNA sample with a known ratio of the polynucleotides is equivalent to the weighted sum of the spectra of individual polynucleotides, indicating that the difference in base stacking interactions produces negligible spectral effects. The variability of nitrogen K-edge spectra in these samples and in protein may be useful for chemically specific imaging using X-ray microscopes.

Introduction

X-rays have been important for understanding the structure of DNA since the early diffraction analysis of nucleosides [1]. Although many classic studies have been done using diffraction, the complementary technique of X-ray absorption has seen relatively little application with DNA. X-Ray Absorption Near-Edge Spectroscopy (XANES), which measures the X-ray absorption cross-section as a function of incident photon energy, is a very useful probe of chemical systems. X-ray absorption studies of platinum have been successfully performed to investigate the nature of binding of *cis*- and *trans*-dichlorodiammine platinum (II) to calf thymus DNA [2].

Earlier examination of N_2 by X-ray absorption spectroscopy revealed the excitation of (in order of increasing energy) a π^* state, a Rydberg series, doubly excited states and finally a shape resonance [3]. These results are consistent with lower resolution photoemission experiments [4,5]. Within the π^* resonance, vibrational structure in accord with the Franck-Condon principle was observed [3]. Other small, nitrogen containing molecules such as pyridine [6] show both σ^* and π^* transitions near the K-edge in their X-ray absorption spectra. Because these transitions possess different angular dependencies, they are useful for discerning the orientation of molecules on surfaces [6–8]. Dynamical processes of core hole states of nitrogen have recently been investigated and are found to be independent of chemical bonding [9]. Other effects can alter the X-ray absorption spectra of nitrogen such as inductive effects of peripheral substitution, substitution at nitrogen sites, hydrogen bonding, as well as subtleties involved in π bonding. In a related system, greater formal oxidation state of sulfur in various compounds was shown to blue shift the energy position [10–12] and enhance the magnitude [11] of the sulfur $1s \rightarrow 3p$ resonance. The increased covalency of nitrogen-carbon bonds over sulfur-carbon bonds results in more complicated X-ray spectra of the nitrogen containing compounds.

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In this report, we investigate the XANES spectra of DNA, polynucleotides, nucleotides, the corresponding bases (including uracil) and two halogen-substituted nucleotides. Several aromatic and saturated nitrogen containing molecules are also studied. Study of all these molecules allows a variety of chemical effects to be investigated. Generally, the π^* resonances are found to be sensitive probes of the nitrogen chemistry as these resonances are variable for different purines (and different pyrimidines). The π^* resonance contains multiple peaks which are probably associated with the different nitrogen sites in the aromatic rings of purines and pyrimidines. The glycosidic substitution at nitrogen ring sites sometimes (but not always) strongly affects the aromatic electronic structure, whereas alkyl substitution at carbon ring sites produces small spectral effects. Oxygen substitution shifts the lowest π^* resonances towards higher energy (by ~ 1.5 eV for two exocyclic carbonyl groups). The small shifts produced by halogen substitution suggest that shifts produced by the exocyclic carbonyl groups arise primarily from the disruption of the aromatic ring system, rather than inductive effects. In addition, spectral variations are observed upon complementary hydrogen bonding of the deoxyguanosine-deoxycytidine (dG-dC) pair. The weighted sum of individual polynucleotides reproduces the spectra of a DNA sample, illustrating that different base-stacking interactions produce negligible spectral effects. For imaging considerations, the spectrum of a protein is contrasted with these spectra. The technology of soft X-ray facilities is changing rapidly and X-ray microscopes are being planned for the Advanced Light Source (ALS) at Berkeley, CA.

Experimental procedures

All the XANES experiments for this study were performed using AT&T Bell Laboratory's Dragon soft-X-ray beam line located at the National Synchrotron Light Source, Brookhaven National Laboratory [13]. The energy of the electrons in the ring was 745 MeV, and the beam current was between 100–800 mA. The pressure in the sample chamber was on the order of 10^{-8} to 10^{-9} torr. A cryopump and a turbomolecular pump were used to maintain high vacuum conditions. A monochromator with a grating made of silica with gold plating was used to scan the proper energy range. The grating used for this experiment was 600 lines/mm. Spectra were recorded from about 60 eV below to about 60 eV above the nitrogen K-edge (409.9 eV), with the monochromator energy resolution of 140 meV (at full width half maximum). A channeltron electron yield detector was used in this experiment. X-ray fluorescence detection was also employed; the corresponding spectra are useful for consistency checks with the electron yield spectra, but are not shown here, except for the case of the substituted pyridine sample, because they suffer from saturation effects.

The biological sample suite consisted of the five nucleic acid bases, the corresponding nucleotides (2′-deoxyadenosine-5′-monophosphate, sodium salt; 2′-deoxyguanosine 5′-monophosphate, sodium salt hydrate; 2′-deoxycytidine 5′-monophosphoric acid, monohydrate; 2′,3′-dideoxythymidine-5′-triphosphate, trisodium salt monohydrate; and 2′-deoxyuridine-3′-monophosphate, sodium salt), the two polynucleotides

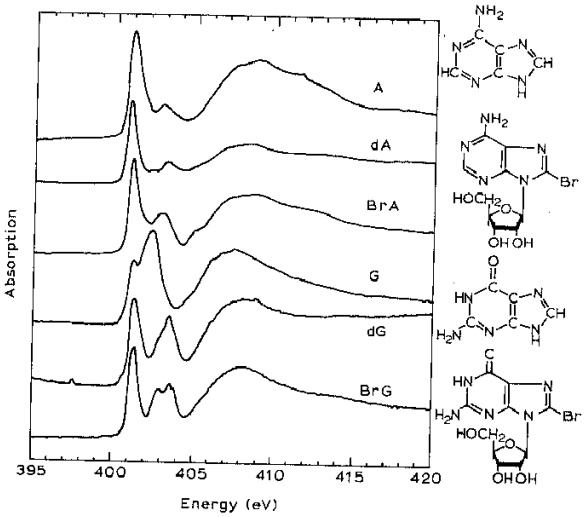

Fig. 1. Nitrogen K-edge XANES spectra of the two purine bases, their corresponding deoxyribose nucleotides and brominated nucleosides (some structures are given). Blue shift of the π^* bands (~ 401 eV) with increased oxygen substitution is seen here. Similar energies of the major π^* resonances between the brominated samples and the corresponding nucleotides suggest that the disturbance of the aromatic ring system is probably the most dominant cause for shifts with increased oxygen numbers.

(poly(dAdT) and poly(dGdC)), two brominated nucleosides (8-bromoadenosine 3′:5′-cyclic monophosphate, and 8-bromoguanosine 3′:5′-cyclic monophosphate, sodium salt) calf thymus DNA sample and one protein (albumin) sample. Other samples include a substituted pyridine, a pyrrolic aromatic and a saturated amine. The sample poly(dGdC) was obtained from Pharmacia. Poly(dAdT), nucleotide ddT and the DNA sample were obtained from CalBiochem. Nucleotides dA, dU and the brominated nucleosides were obtained from Sigma, and the rest of the samples were obtained from Aldrich. All the samples were used without further purification. The polynucleotide samples had alternating bases and were double stranded. The bases, nucleotides, brominated nucleotides and protein sample were in powder forms, and the rest of the samples were similar in appearance to small cotton-wool pieces. The powder samples were first ground into fine particles in a mortar and pestle. All the samples were mounted on a gold-plated sample holder by means of nitrogen-free double sticking tape, or rubber cement.

Results and Discussion

Fig. 1 shows the nitrogen K-edge XANES spectra of the two purine bases of the nucleic acids, the corresponding nucleotides and the brominated nucleosides (the chemical structures of the bases, and brominated nucleoside, are shown next to the corresponding spectra). Fig. 2 plots similar spectra of the three pyrimidine bases, and their corresponding nucleotides (the chemical structures of the three bases are also given). In all these samples, the lower energy resonances (400 eV < E < 403 eV) result from 1s to π^* electronic transitions, while the broader, higher energy resonances (around 407 eV) result from transitions to the σ^* levels. The position and shape of the π^* resonances are seen to vary appreciably for the different compounds. The pyrimidine bases show similar and slightly more structured σ^* resonances than the purines. The different purine and pyrimidine bases, their nucleotides and the polynucleotides exhibit a wide variation of chemical bonding and substituents. The impact of these chemical variations on the nitrogen XANES spectra can readily be observed.

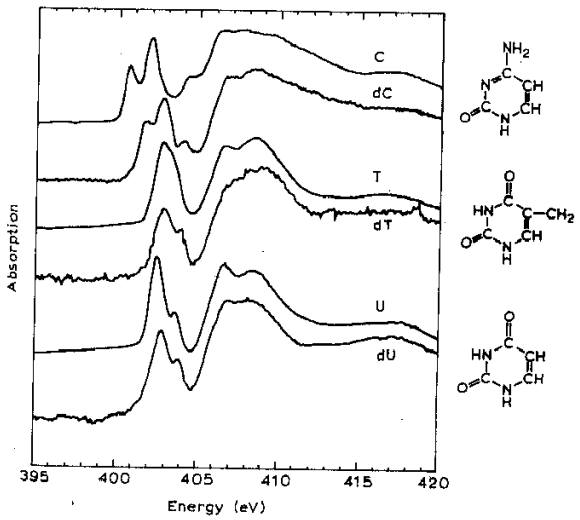

Fig. 2. Nitrogen XANES spectra of the three pyrimidine heterocyclic bases and their corresponding nucleotides. The blue shift of the π^* resonances with increased oxygen substitution is seen. Glycosidic C-N bond formation produces spectral effects in some cases.

TABLE I

Energies of the largest π^* resonances in the nitrogen XANES spectra of some bases, nucleotides and halogen-substituted nucleosides

Samples	Peak position (eV) (±0.15 eV)
Adenine (A)	401.02
dA	400.95
BrA	401.10
Guanine (G)	401.17/402.32
dG	401.31
BrG	401.33
Cytosine (C)	400.68/402.00
dC	401.66/402.74
Thymine (T)	402.76
ddT	402.85
Uracil (U)	402.49
dU	402.79

Adenine has no exocyclic carbonyl groups, guanine and cytosine each have one; whereas thymine and uracil each have two exocyclic carbonyl groups. We observe a shift in the mean position of the π^* resonances towards higher energy with increased oxygen substitution (Figs. 1 and 2). Table I shows the energy of the most intense π^* resonance of the bases, the corresponding nucleotides, and the two halogenated nucleosides. The uncertainty in these measurements is about twice the resolution of the apparatus. Note that in the cases of G, C and dC, the stronger π^* resonance is at a higher energy, immediately following the first weaker π^* resonance. In these cases both the peak positions are noted.

The energy shift between the major peaks of adenine and thymine is 1.80 eV. A large blue shift is also observed in the corresponding nucleotides. Blue shifts in peak positions with increased oxygen substitution have been seen in sulfur. In the sulfur case, the oxygen is bonded directly to the sulfur and the difference in peak position for sulfur in the 0 and +6 formal oxidation states is ~10 eV [10,11]. In our case, however, oxygen is not directly bonded to nitrogen, and there are two plausible reasons for the blue shift with carbonyl substitution. The inductive effect (less enhanced than the case of oxygen bonded to sulfur) from the

electron withdrawing exocyclic carbonyl groups, and the disruption of the aromatic ring system from carbonyl substitution could cause this shift. Brominated adenosine and brominated guanosine were next studied in order to test for the dominant reason. Halogen substitution was chosen since it has a stronger inductive effect and a smaller disruptive effect on the aromatic systems than the carbonyl group. The halogenated nucleosides show very small blue shifts from the corresponding nucleotides, 0.20 eV or less. The direction of the spectral shift of the lowest energy peak with electron withdrawing bromine substitution is towards higher energy, as expected. Note also that bromination causes a splitting of the higher energy π^* peak for guanosine. The magnitude of the shift induced by bromine is much smaller than that produced by the carbonyl groups. This suggests that the blue shift with exocyclic carbonyl substitution is primarily due to the disturbance of the aromatic ring system. Other tautomeric forms of the bases are far less prevalent [1], and are expected not to influence the corresponding spectra.

The nucleotides differ from bases by substitution of a (phosphate-ester-containing) sugar on one of the ring nitrogens; the substitution is called a β-glycosidic C-N linkage (Fig. 1) [1]. Somewhat surprisingly, this C-N glycosidic substitution sometimes produces large differences between the XANES spectra of the bases and the nucleotides (Figs. 1 and 2), and occur for both purines and pyrimidines. The differences are largest for guanine. Apparently, the glycosidic C-N linkage produces subtle changes on the ring nitrogen which may or may not impact the π system. Perhaps the C-O of the glycosidic carbon can interact with the π system of the ring. Crystal structure changes may also be an important determinant. Thymine and uracil are chemically quite similar; thymine, unlike uracil, has a methyl substitution at a (ring) carbon site (see Fig. 1). The XANES spectra of thymine and uracil are also very similar to each other; the spectra of dideoxythymidine (dT) and deoxyuridine (dU) are similar to each other as well. Thus, substitution of a methyl group for hydrogen at a ring carbon site has little effect on the nitrogen spectra.

Fig. 3 plots the XANES spectra of three different nitrogen compounds, the respective structures shown next to the spectra. The polymer contains a pyridine subunit, carbazole a pyrrole subunit, and hexahydrotriazine is a saturated amine. Hexahydrotriazine, being a saturated molecule, exhibits only a σ^* nitrogen resonance at a higher energy position ($E = 407.3$ eV). In the spectra of aromatic pyridine and pyrrole, on the other hand, both π^* (at energies between 400.0 and 403.3 eV) and σ^* resonances (at around 408.0-410.5 eV) are observed. Large energy shifts relative to peak widths are observed in the π^* resonances of the two aromatic compounds. In the pyrrole structure, resonance effects dominate, while in the pyridine, elecronegativity effects dominate. In a pyrrole structure, the lone pair of electrons of the nitrogen is involved in the π aromatic ring system, and there is a net loss of electron density at the nitrogen site, while in the six-membered pyridine structure, the lone pair of electrons of the nitrogen is unshared in a sp^2 orbital. Pyridine, therefore, is more basic than pyrrole, and consequently, the π^* resonance of the pyridine spec-

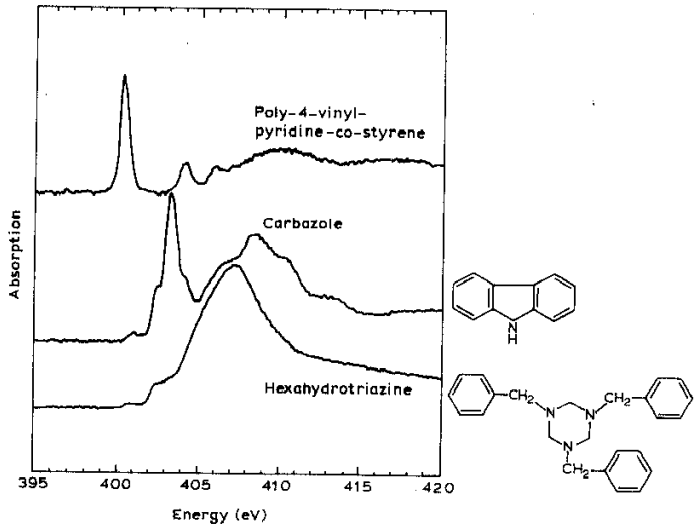

Fig. 3. Nitrogen XANES spectra of three nitrogen compounds: a pyridine, a pyrrole and a saturated amine (chemical structures of two are given). The pyridine and pyrrole spectra show both a lower energy $1s \rightarrow \pi^*$ and a higher energy $1s \rightarrow \sigma^*$ electronic transitions. The spectrum of the saturated amine, on the other hand, shows only a $1s \rightarrow \sigma^*$ electronic transition. The pyrrole nitrogen π^* resonance occurs at higher energy than that of pyridine nitrogen in accord with the greater basicity of pyridine nitrogen.

trum is red shifted compared to that of the pyrrole, by about 3.1 eV.

Table II shows a comparison between the energy positions and the peak widths (full widths at half maximum) of the π^* resonances of the spectra of adenine, thymine, and pyridine. The widths of adenine and pyridine are comparable, whereas thymine has two broader π^* resonances. Multiple large π^* resonances are probably associated with multiple nitrogen sites. The two π^* resonances may correspond to ring nitrogens with and without attached hydrogens, corresponding to pyrrolic and pyridinic nitrogens, respectively. Fig. 3 shows a single dominant π^* resonance for molecules with one nitrogen site. N_2 also shows only one π^* resonance [2].

Fig. 4 shows four separate plots. The first part, (a), compares the weighted sum (dashed line) of the spectra of dA and dT (1 : 1) with the spectrum (solid line) of the double-stranded polynucleotide dAdT sample. The spectral sum of the individual nucleotides, dA and dT is very similar to the spectrum of poly(dAdT). Part (b) of Fig. 4 compares the weighted sum (dashed line) of the spectra of dG and dC (1:1) and the spectrum (solid line) of the polynucleotide dGdC. For this case, the overall features of the sum of the spectra of dG and dC are similar to the features of the spectrum of poly(dGdC); nevertheless, there are distinct differences between the two. The energy difference between the first two peaks is 2.10 eV in poly(dGdC) and 1.50 eV in the spectral sum. An overall shift in the spectra would not affect the energy difference between two consecutive peaks in the spectra. The spectral sum also exhibits a deeper valley than the nucleotide, at 404.6 eV. The two spectra appear to be slightly shifted as well, by 0.20 eV.

Complementary hydrogen bonding accompanies the formation of the poly(dGdC). Three hydrogen bonds form between the nucleotide pairs dG and dC in poly(dGdC). The difference between the sum of spectra of dG and dC and of the spectrum of the poly(dGdC) is likely produced by complementary hydrogen bonding. However, the two hydrogen bonds which form between dA and dT do not produce spectral effects. For reasons which are not clear, Figs. 1 and 2 show that G and C are very sensitive to chemical effects such as the glycosidic bond formation, whereas

TABLE II

Energies and widths of the π^* resonances in the nitrogen XANES of several nitrogen compounds

Sample	Energy position (eV)	Peak width (eV)
Adenine	401.02	0.67
Thymine	402.76/403.2	1.2/1.1
Pyridine	400.15	0.58

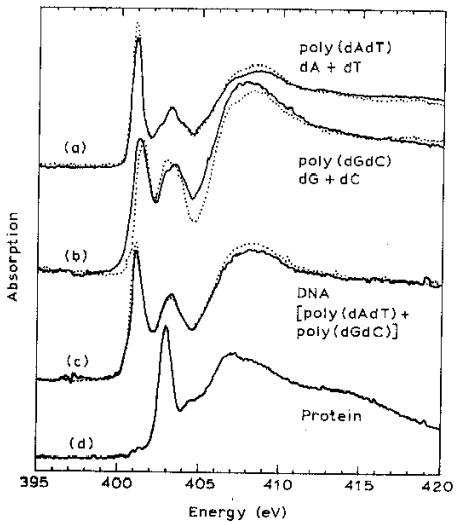

Fig. 4. Nitrogen XANES spectra for various samples. Part (a) shows that the spectrum of double-stranded poly(dAdT) (solid line) is very similar to the sum of spectra of dA and dT (dashed line). In part (b), the spectra of poly(dGdC) (solid line) differs from the sum of spectra (dashed line) of dG and dC probably due to hydrogen-bonding effects. The spectrum of DNA (solid line) in part (c) equals a weighted sum of spectra (dashed line) of poly(dAdT) and poly(dGdC). The spectrum of the protein, albumin, shown in part (d) differs from that of DNA.

A and T are relatively insensitive. The hydrogen bonding in the guanine-cytosine pair is stronger than that in the adenine-thymine pair [1]. The observed sensitivity and the known stronger hydrogen bonding account for the larger spectral impact of complementary hydrogen bond formation of the dGdC pair than the dAdT pair.

Calf thymus DNA was next studied. This particular DNA is known to contain 42% dGdC base pairs and the rest of dAdT. The third part of Fig. 4(c), shows comparison between the spectra of the nucleic acid, and of the simulated sum of the known ratios of the corresponding polynucleotides. It is clearly evident that the two spectra are nearly identical. The polynucleotides have the same base pairing and hydrogen bonding as DNA. The only difference corresponds to nearest neighbors. In spite of close proximity of bases, the differential nearest neighbor interactions, or base stacking effects are apparently small, and have negligible spectral impact.

Part (d) of Fig. 4 shows a plot of the protein albumin. The primary π^* resonance occurs at 402.9 eV, and differs significantly from the DNA. XANES spectroscopy could be used for biological imaging applications, for instance, for differentiating between the DNA and proteins, by using their characteristic wavelengths for the π^* resonances. X-ray microscopes for imaging purposes are being developed at the Advanced Light Source.

Conclusions

DNA, polynucleotides, nucleotides and their corresponding bases have been examined using nitrogen XANES methods. The $1s \rightarrow \pi^*$ resonances are found to be particularly sensitive to the detailed chemical nature of the nucleic acid bases. Halogen substitution at carbon sites produces small blue shifts in the lowest π^* resonance, while increased exocyclic carbonyl substitution produces correspondingly large blue shifts. Thus, the spectral effects of the carbonyl group are most likely due to the disturbance of the aromatic system. The pyrrole nitrogen π^* resonances is at a higher energy than pyridine nitrogen in accord with the greater basicity of pyridine nitrogen. Surprisingly, glycosidic substitution on ring nitrogen sites is found to produce large spectral effects in some cases (for guanine and cytosine). Comparison of the sum of nucleotide spectra with the spectra of double stranded polynucleotides illustrates that distinct spectral variations accompany the formation of complementary hydrogen bonding in the poly(dGdC) case. XANES spectroscopy could be useful for imaging biological molecules, differentiating nucleic acids from proteins with the help of the characteristic π^* resonance wavelengths.

Acknowledgements

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