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Conformation Examination of Uridine Diphosphoglucose Using Lanthanide–Nitrilotriacetate Chelates as Shift Probes†

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ABSTRACT: A ^{13}C NMR study utilizing the lanthanide-induced shift (LIS) technique was carried out on uridine diphosphoglucose (UDPG) in order to determine the solution conformation. The neutral, water soluble nitrilotriacetate chelates, Ln(NTA), were used in preference to the bare ions due to the potency of the latter in hydrolyzing the UDPG. In addition to the primary binding site at the pyrophosphate moiety, secondary binding to the uracil carbonyl groups was observed and found to be a function of the size of the lanthanide ion; the largest ions showed the greatest relative affinity for the carbonyl groups. This effect was overcome by extrapolating the observed shift ratios to zero Ln(NTA)/[UDPG]. The extrapolated shift ratios were relatively constant for a series of five lanthanides indicating the solution conformation of UDPG is insensitive to metal ion size. The conformation of the UDPG was found to be extended so that the uracil carbons as well as carbons 1′, 2′, and 3′ of the ribose are outside of the pseudo-contact shift cone with the remaining carbons inside the cone.

Analysis of ^1H–^1H and ^1H–^{31}P coupling constants and chemical shift data has provided detailed information on the carbohydrate conformation in several nucleoside diphosphohexoses (Sarma et al., 1973; Lee & Sarma, 1976). Based on the absence of significant hexose shifts relative to the hexose 1-phosphates, indicating negligible ring current interactions in adenosine diphosphoglucose (ADPG),[1] it was concluded that the nucleotide and hexose moieties are relatively distant. This conclusion is somewhat weaker for uridine diphosphoglucose (UDPG) in which ring current shifts due to the uracil are smaller than those produced by adenine. Thus, the solution conformational structures of UDPG and, more importantly in terms of glycosyl transferase cofactors, its metal ion complexes are uncertain.

Lanthanide-induced shift (LIS) studies have become increasingly popular in probing solution structures of biologically important molecules (Dobson & Levine, 1976). Specific binding of these paramagnetic ions to a small molecule results in magnetic perturbations in NMR active nuclei which depend upon the average solution geometry of the metal–substrate complex. Solution structures have been derived for relatively nonflexible molecules such as 5′-adenosine monophosphate (Barry et al., 1971), cyclic adenosine 3′,5′-monophosphate (Lavallee & Zeltman, 1974), indole-3-acetate (Levine et al., 1974), and L-alanine (Sherry & Pascual, 1977) as well as for conformationally flexible molecules such as dinucleoside phosphates (Barry et al., 1972), phospholipids (Bystrov, 1971), adenosine triphosphate (Transwell et al., 1975), and simple peptides (Levine & Williams, 1975). In the present study, several lanthanide nitrilotriacetate (NTA) chelates have been used to obtain overall conformational information on UDPG. The use of a chelate was required due to the potency of the bare lanthanide ions in hydrolyzing the nucleotide diphosphohexoses

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[1] Abbreviations used: ADPG, adenosine diphosphoglucose; UDPG, uridine diphosphoglucose; LIS, lanthanide-induced shift; NTA, nitrilotriacetate.

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(Nunez & Barker, 1976). The information available using this approach complements the more detailed and less general information from coupling constant studies.

Materials and Methods

Uridine diphosphoglucose (UDPG) was obtained from Sigma. Lanthanide chlorides were prepared from 99.9% pure lanthanide oxides and their solutions standardized against ethylenediaminetetraacetic acid (EDTA) using xylenol orange as an indicator (Lyle & Rahman, 1963). One-to-one lanthanide–nitrilotriacetate complexes were prepared in D_2O, adjusted to ~pH 5 and solid UDPG was added directly to the Ln(NTA) solutions. This procedure eliminates the problem of sample dilution resulting from addition of a Ln(NTA) solution to a UDPG solution. The pH was adjusted to 4 (uncorrected for the deuterium isotope effect) immediately preceding NMR analysis.

Proton-decoupled carbon-13 spectra were recorded at 7 °C on a Varian XL-100 Fourier transform spectrometer locked on the D_2O solvent and interfaced to a Nova 1210 computer. Typically, 4K spectral data points were gathered over a 4000-Hz sweep width resulting in a spectral resolution of ±0.04 ppm. The lanthanide induced shifts (LIS) in the uridine diphosphoglucose carbon resonances were measured from the internal reference tetramethylammonium ion and are reported relative to diamagnetic La(NTA)–UDPG and Lu(NTA)–UDPG. Both diamagnetic complexes produce negligible chemical shifts perturbations from free uncomplexed UDPG. Assignments of the ^{13}C resonances of UDPG were made on the basis of comparison with results for UMP (Mantsch & Smith, 1972) and α-D-glucose 1-phosphate (Nunez et al., private communication).

Initial LIS experiments were attempted using the bare unchelated aquo-ions as shift probes but these solutions gave evidence of considerable (~5–10%) hydrolysis of UDPG into UMP and glucose 2-phosphate (Nunez & Barker, 1976) after 5–6 h of NMR scan time. The hydrolysis further complicated the experiments by lowering the pH during data accumulation. Subsequent experiments were attempted using Ln(EDTA) and Ln(NTA) chelates as shift probes. The EDTA chelates were ineffective largely due to solubility limitations at low pH, while the Ln(NTA) chelates proved quite advantageous. The shifts produced by the latter chelates were similar in magnitude to those produced by the uncomplexed aquo ions while the problems associated with UDPG hydrolysis were eliminated. The Ln(NTA)-UDPG showed no evidence of hydrolysis after 30–36 h at pH 4 and 7 °C.

Results

The frequency shifts produced in NMR active nuclei as a result of lanthanide ion binding to a specific site on a substrate may result from a through-space dipolar interaction (pseudo-contact shift), a direct delocalization of unpaired electron spin density from the metal to the nuclei (contact shift), or from a sum of these two mechanisms. Frequency shifts arising from pseudo-contact origins are related to the average dynamic structure of the lanthanide–substrate complex (Bleaney, 1972):

$$\Delta\nu/\nu = \frac{g^2\beta^2 J(J+1)(2J-1)(2J+3)D_z}{60kT^2}\,\frac{3\cos^2\theta - 1}{r_i^3}$$

The first term contains magnetic constants characteristic of the lanthanide ion f orbital population and the ligand field term, D_z, while the second contains the needed geometrical information, i.e., r_i is the distance between nucleus i and the metal ion and θ describes the angle between each metal–nucleus vector and the principal symmetry axis of the ligand field. This simplified equation assumes rapid exchange yielding a Ln–UDPG complex with effective axial symmetry (Horrocks, 1974). By expressing the lanthanide-induced shifts within a given Ln–UDPG complex as ratios, the equation is further simplified to yield the necessary geometrical contraints. As the shift ratios are independent of the particular lanthanide ion used, providing homologous complexes are formed, they also become a sensitive measure of UDPG structural alterations caused by metal ion size or ligand field requirements.

Shifts produced as a result of electron spin delocalization in this system are expected to be small. The phosphorus shifts in the Ln–ATP complexes are dominated by contact interactions but fall off away from this nucleus and become negligible at the ribose 5′ protons (Tanswell et al., 1975). The internal consistency of all shift ratios in UDPG as discussed below using the glucose C-1 carbon as the reference further suggests that contact interactions are small.

The use of the lanthanide ions as conformational probes is also predicated on the assumption of one binding site in the molecule being probed. The effect of secondary binding in a large biological molecule can be particularly misleading if the secondary site is sufficiently remote from the primary binding site in which case the shifts of nuclei close to the secondary binding site may be dominated by the more closely bound ions. In order to minimize such effects, lanthanide ion induced shift ratios were plotted as a function of the [Ln(NTA)]/[UDPG] ratio and extrapolated to zero lanthanide ion concentration.

The results ranged from shift ratios which were essentially independent of lanthanide concentration for Yb(NTA) and Er(NTA), to a dramatic dependence on concentration for Pr(NTA), with Dy(NTA) and Tb(NTA) falling closer to the Yb(NTA) limit. Extrapolated shift ratios relative to glucose C-1 are given in Table I and representative Pr(NTA) data in Table II. The reasonably close agreement of the extrapolated shift ratios in Table I indicates that contact contributions to the shifts are not significant and the conformational differences between the various Ln(NTA)–UDPG complexes are minor.

There are two reasons for expecting significant effects due to secondary binding in the present study. First, the use of the neutral Ln(NTA) complexes rather than the bare ion will reduce the relative affinity for the negatively charged pyrophosphate moiety which constitutes the primary binding site. Secondly, the carbonyl groups of uracil appear to be reasonably good ligands for the Ln(NTA) complex. The first factor noted above has been encountered in previous studies of the Ln(EDTA) complexes which bind significantly only to the dianion form of monophosphate groups (Dobson et al., 1974). The evidence for secondary binding to the uracil base is supported by the fact that the shift ratios for the uracil ring carbons exhibit the most pronounced dependence on the lanthanide ion concentration. For the Pr(NTA) case, the shifts resulting from secondary binding to the uracil carbonyls appear to be opposite in direction to the shifts produced by the phosphate bound lanthanide, reflecting the fact that the uracil moiety is outside of the shift cone of the primary lanthanide. In interpreting the uracil shifts, however, it should be noted that, if the lanthanides are interacting directly with the uracil carbonyl groups, a significant contact contribution from the secondary lanthanide may be present.

Differences in the mode in which the various lanthanide ions bind to carboxylate and phosphate anions have been noted in a number of systems (Sherry & Pascual, 1977; Levine et al., 1974; Barry et al., 1974; Dobson & Levine, 1976, and references therein). The present results indicate that the stability

TABLE I: Lanthanide-Induced Shift Ratios in the Carbon Resonances of Uridine Diphosphoglucose.[a]

carbon resonance	Yb(NTA)	Er(NTA)	Dy(NTA)	Tb(NTA)	Pr(NTA)
UC2	−0.11	−0.09	−0.20	−0.23	−0.18
UC4	−0.10	−0.09	−0.28	−0.30	−0.21
UC5	−0.11	−0.13	−0.26	−0.36	−0.18
UC6	−0.06	−0.04	−0.09	−0.26	0.07
GC1	1.00	1.00	1.00	1.00	1.00
GC2	0.30	0.27	0.66	0.38	0.39
GC3	0.00	−0.04	0.07	0.05	0.20
GC4	0.01	−0.03	0.06	0.01	0.16
GC5	0.30	0.24	0.13	0.10	0.36
GC6	0.12	0.09	0.06	0.01	−0.08
RC1′	−0.11	−0.08	−0.09	−0.17	0.13
RC2′	−0.19	−0.18	−0.08	−0.18	0.02
RC3′	−0.13	−0.10	0.02	−0.09	0.13
RC4′	0.01	0.08	0.20	0.03	0.55
RC5′	0.54	0.61		0.73	1.42

[a] The shift ratios were extrapolated to zero Ln(NTA) concentration to minimize shift contributions from secondary binding sites.

TABLE II: Pr(NTA)-Induced Shifts in Uridine Diphosphoglucose at pH 4.[a]

carbon resonance	[Pr(NTA)]/[UDPG] ratio			
	2.56	1.25	0.67	0.56
UC2	0.01	−0.02	−0.09	−0.11
UC4	0.00	−0.04	−0.12	−0.13
UC5	0.02	−0.03	−0.10	−0.11
UC6	0.21	0.18	0.13	0.12
GC1	1.00	1.00	1.00	1.00
GC2	0.37	0.40	0.40	0.39
GC3	0.20	0.19	0.19	0.20
GC4	0.18	0.16	0.15	0.15
GC5	0.37	0.39	0.36	0.34
GC6	0.07	0.03	−0.02	−0.03
RC1′	0.26	0.23	0.18	0.18
RC2′	0.18	0.07	0.05	0.04
RC3′	0.24	0.20	0.17	0.16
RC4′	0.45	0.46	0.50	0.60
RC5′		1.25	1.32	1.35

[a] The shift ratios are reported for a constant 0.1 M Pr(NTA) concentration and variable UDPG concentrations.

ratio of the primary phosphate binding site to the secondary carbonyl site(s) decreases in the order Yb ~ Er > Dy ~ Tb ≫ Pr. These results suggest the importance of ion size in determining the relative affinity of the Ln(NTA) for the different sites. The fact that the smallest lanthanides appear to have the most specific affinity for the pyrophosphate is not surprising given the greater stability of Mg^{2+}-nucleotide complexes compared with Ca^{2+}-nucleotide complexes (Khan & Martell, 1962). It is worth emphasizing that the strongest dependence of shift ratios on lanthanide ion concentration was obtained with praseodymium, one of the most commonly used lanthanides in such studies.

In addition to differences in affinity for the primary and secondary binding sites, there are small but significant differences in the extrapolated shift ratios obtained for the various lanthanides (Table I). These differences may reflect minor changes in the structure of the Ln(NTA)-UDPG complex. A relatively small perturbation in the conformation of the pyrophosphate moiety can significantly affect the position of the remote carbons relative to the lanthanide ion and the principal symmetry axis. It has been reported that the binding of Eu^{3+} or La^{3+} to the pyrophosphates of NAD and AppA causes conformational changes relative to the metal free dinucleotide structures (Bayley & Debenham, 1974). Our LIS data may reflect these same conformational changes which seem to depend upon the metal ion size (i.e., extrapolated shift ratios for the ribose C-5′ carbon, which was found to be most sensitive to the specific lanthanide ion used, follow the order Pr ≫ Tb > Er > Yb).

Qualitative Conformational Conclusions. Despite the differences which exist, a general picture emerges from these studies in which the lanthanide ion binds primarily to the pyrophosphate moiety approximately midway between the glucose C-1 and ribose C-5′. The glucose moiety is located within the shift cone defined by $3\cos^2\theta - 1$ with C-3 and C-4 sufficiently close to the edge so that in one case the shift is in the opposite sense relative to C-1 (Table I). Similarly, while the C-4′ and C-5′ carbons of ribose are located within the shift cone, the ribose C-1′, C-2′, and C-3′ carbons, as well as all of the uracil carbons are entirely outside of the cone. These effects are illustrated in the spectra obtained with Yb(NTA), Lu(NTA), and Tb(NTA), with the opposing shifts of the uracil and glucose carbons particularly apparent in the latter (Figure 1). These results require that the average UDPG conformation is extended. The overall structure of the UDPG relative to the shift cone of the lanthanide is schematically illustrated in Figure 2. The above conclusion is consistent with the fact that the chemical shifts observed for UDPG are very close to the sum of the shifts for UDPG and α-D-glucose 1-phosphate, suggesting an extended conformation since a closer association would probably result in ring current shifts (Sarma et al., 1973; Lee & Sarma, 1976).

The above conformational results are qualitatively similar to those obtained from ^1H and ^{31}P NMR studies of lanthanide ion ATP complexes (Tanswell et al., 1975). In that case, the orientation of the shift cone is such that all of the phosphorus atoms and none of the protons are included. Alternatively, studies with several nucleotide monophosphates (Dobson et al., 1974; Lavallee & Zeltman, 1974; Dobson & Levine, 1976, and references therein) indicate that essentially the entire molecule is contained in the shift cone of the lanthanide. Results with the diphosphate are therefore intermediate between those for the monophosphate and triphosphate cases with several of the carbons contained within the shift cone.

Quantitative Conformational Results. Given the essential flexibility of the UDPG molecule, any unique conformational conclusions for the Ln(NTA)-UDPG complex must be viewed with skepticism. The primary value of the present study is in establishing the general conformational features summarized

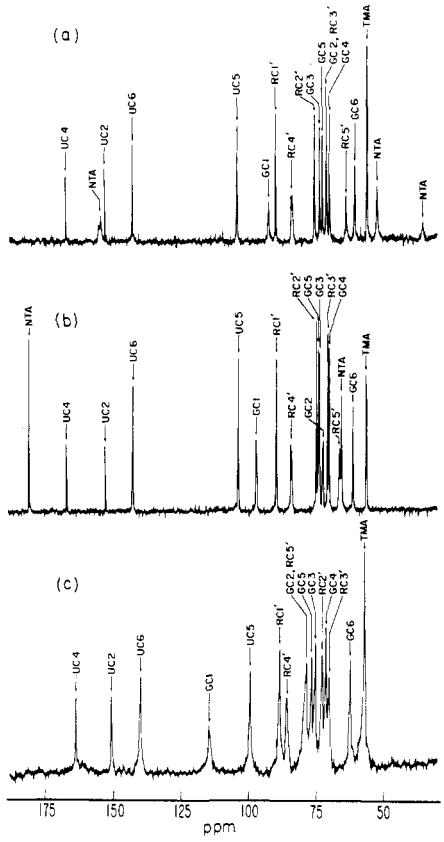

FIGURE 1: Proton decoupled ^{13}C NMR spectra of UDPG in the presence of Ln(NTA) complexes: (a) 0.102 M UDPG, 0.1 M Yb(NTA); (b) 0.19 M UDPG, 0.1 M Lu(NTA); (c) 0.093 M UDPG, 0.1 M Tb(NTA). All samples contained 0.2 M tetramethylammonium bromide as an internal standard. Spectra were run at 7 °C, pH 4.0. Uracil carbons are indicated by UC, ribose carbons by RC, and glucose carbons by GC using standard numbering. The tetramethylammonium resonance appears as a triplet denoted by TMA. NTA peaks are observable in a and b, but not c, due to excess line broadening induced by the Tb^{3+}. In the YB(NTA) complex, the NTA peaks for both the carboxyl and methylene groups appear as two resonances each, indicating slow exchange between inequivalent sites.

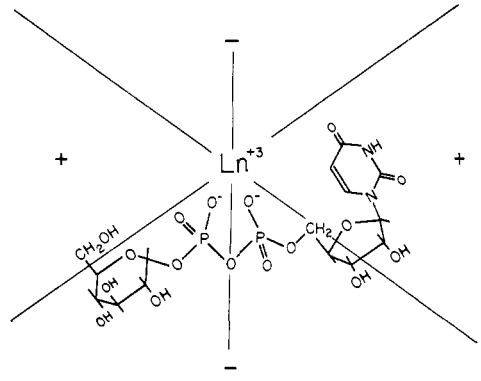

FIGURE 2: Schematic illustration of the relative orientation of the Ln(NTA)-UDPG complex indicating approximate positions of the carbons relative to the shift cone defined by $3\cos^2\theta - 1$. As a result of the two-dimensional representation, some distortion is introduced so that the cone boundary actually occurs between ribose C-3′ and C-4′ rather than between the ribose C-3′ and C-2′. The principal symmetry axis from which θ is measured is assumed to pass through the central oxygen of the pyrophosphate.

TABLE III: Comparison of the Calculated and Experimental Shift Ratios in the Ytterbium-Uridine Diphosphoglucose Complex.

nucleus	experimental shift ratio	calculated shift ratio
UC2	−0.11	−0.07
UC4	−0.10	−0.12
UC5	−0.11	−0.14
UC6	−0.06	−0.05
GC1	1.00	0.86
GC2	0.30	0.32
GC3	0.00	0.08
GC4	0.01	0.12
GC5	0.30	0.28
GC6	0.12	0.18
RC1′	−0.11	−0.05
RC2′	−0.19	−0.27
RC3′	−0.13	−0.05
RC4′	0.01	0.26
RC5′	0.54	0.67

in Figure 2. A somewhat more specific fit of the data using the Yb(NTA) shift results was attempted using a modified version of the program, PDIGM, which allows movement of the principal axis of symmetry (Sherry & Pascual, 1977). First, a search for the best lanthanide binding position and direction of the principal axis of symmetry vector was made relative to the crystal atomic coordinates of uridine monophosphate (Shefter & Trueblood, 1965) and α-glucose 1-phosphate (Beevers & Maconochie, 1965). Both calculations gave best fits for a lanthanide position near the individual phosphates with the symmetry axis vector pointing toward one of the remaining free phosphate oxygens. The two calculated lanthanide complexes were next connected by making the two symmetry axis vectors colinear, thereby creating the bridging oxygen of UDPG and a unique metal location which fits all data. This Ln-UDPG structure displays a bridging P-O-P bond angle of ~160° and two Ln-P distances of 4.0 and 5.4 Å, putting the lanthanide ion closer to the glucose moiety.

The calculated shift ratios were further refined by allowing minor adjustments in rotationally flexible portions of the structure. Rotation of the entire glucose or uridine moieties about their respective P-O or O-R bonds did not improve the agreement between the calculated and experimentally measured shift ratios. However, a 20° counterclockwise rotation of the nucleoside about its C-5′-C-4′ bond and a 40° clockwise rotation of the uracil ring about the glycosidic bond significantly improved the fit. The resulting calculated shift ratios are compared with the experimental shifts produced by Yb(NTA) in Table III. The agreement between the two sets of data is remarkably good. The direction of each shift is correctly predicted and the largest deviations are observed for those nuclei lying near the edge of the shift cone, i.e., RC4, RC3, GC4, and GC5.

It should be noted that the assignment of the closely spaced glucose C-3 and C-5 resonances in the Lu(NTA)-UDPG complex, which was used as a reference in computing the lanthanide induced shifts, is based on a comparison of the computed and observed shift ratios (Table III). Although in the model compound α-D-glucose 1-phosphate, C-3 is downfield relative to C-5, it is found that, as the charge on the phosphate group is reduced (lowering the pH), the shift difference between C-3 and C-5 decreases to 0.2 ppm at pH 4.0 (H. Nunez, private communication). Thus, it is not unreasonable that complexation with Lu(NTA) would further shift C-5 downfield relative to C-3. We note in support of this conclusion that, in uncomplexed UDPG, C-3 and C-5 exhibit the same chemical shift.

Conclusions

The lanthanide ion probe method appears to be particularly well suited for providing overall conformational information

which complements the detailed information obtained from an analysis of scalar coupling constants. The use of NTA chelates was found to resolve the hydrolysis problem which exists with the bare ions for the case of UDPG (Nunez & Barker, 1976) as well as minimizing the conformational perturbation induced by the +3 charge of the lanthanide. Alternatively, it is probable that the Ln(NTA) complex exhibits significantly less specificity for negatively charged groups so that secondary binding may pose a greater problem than with the bare ions. As in the present case, such effects can be minimized by extrapolation of the shift ratios to zero lanthanide ion concentration. Even in cases where the bare lanthanides can be used, however, the possibility of secondary binding may be significant in typical biological molecules containing many potential binding sites. For this reason, the lanthanides which produce the largest shifts are likely to give the most reliable data for nuclei remote from the primary binding site. The broadening problem appears to be less severe for ^{13}C studies due to the larger range of chemical shifts.

It is interesting to note that in the present study the best data were obtained with Ln(NTA) complexes of the later lanthanides. In contrast, Dobson & Levine (1976) have found that shifts obtained with Ln(EDTA) complexes where Ln is the second half of the series are difficult to observe because of excessive broadening; it was concluded that fast exchange could not be assumed for these complexes so that they are not useful as conformational probes. Whether these differences reflect the difference between NTA and EDTA as lanthanide chelates or differences between the systems studied is unclear.

Finally, the present study has provided a clear picture of UDPG as existing primarily in an extended conformation in solution, a result consistent with previous chemical shift data. The solution conformation of UDPG is of particular interest in view of proposals for a direct base–hexose interaction in several enzyme-catalyzed reactions (Szulmajster, 1961; Budowsky et al., 1966). It is interesting to note that the closely related structure for which crystallographic data are available, cytidine-5′-diphosphocholine (CDP-choline), adopts a U-shaped conformation in which the choline methyl groups are fairly close to the cytosine base (Viswamitra et al., 1975). Thus, either the substitution of choline for glucose acts as a significant perturbation of the molecular conformation, or the most probable solution conformation of CDP choline differs significantly from the crystalline conformation. The present method of analysis appears ideally suited to resolving this problem.

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