

Hydrogen bond interactions of G proteins with the guanine ring moiety of guanine nucleotides

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(RECEIVED July 9, 1993; ACCEPTED November 1, 1993)

Abstract

We have utilized Raman difference spectroscopy to investigate hydrogen bonding interactions of the guanine moiety in guanine nucleotides with the binding site of two G proteins, EF-Tu (elongation factor Tu from *Escherichia coli*) and the c-Harvey *ras* protein, p21 (the gene product of the human c-H-*ras* proto-oncogene). Raman spectra of proteins complexed with GDP (guanosine 5' diphosphate), IDP (inosine 5' diphosphate), 6-thio-GDP, and 6-¹⁸O-GDP were measured, and the various difference spectra were determined. These were compared to the difference spectra obtained in solution, revealing vibrational features of the nucleotide that are altered upon binding. Specifically, we observed significant frequency shifts in the vibrational modes associated with the 6-keto and 2-amino positions of the guanine group of GDP and IDP that result from hydrogen bonding interactions between these groups and the two proteins. These shifts are interpreted as being proportional to the local energy of interaction (ΔH) between the two groups and protein residues at the nucleotide binding site. Consistent with the tight binding between the nucleotides and the two proteins, the shifts indicate that the enthalpic interactions are stronger between these two polar groups and protein than with water. In general, the spectral shifts provide a rationale for the stronger binding of GDP and IDP with p21 compared to EF-Tu. Despite the structural similarity of the binding sites of EF-Tu and p21, the strengths of the observed hydrogen bonds at the 6-keto and 2-amino positions vary substantially, by up to a factor of 2. The hydrogen bond between the protein and the 6-keto group is stronger for GDP in EF-Tu compared to p21, but this comparison is reversed for bound IDP. Thus, the removal of the 2-amino group from GDP to form IDP affects binding properties distal to the amino site.

Keywords: EF-Tu; G proteins; hydrogen bonding; nucleotide binding; p21; Raman spectroscopy

A variety of key cellular processes such as signal transduction, protein transport, and protein biosynthesis are regulated in vivo by members of the guanine nucleotide binding protein (also termed GTPase) superfamily. These proteins share strong functional and structural homologies (for reviews, see Masters et al., 1986; Wooley & Clark, 1989; Bourne et al., 1990, 1991), and their biochemical activities are tightly regulated by the nature of the bound nucleotide. In general, they are biochemically active ("on") in the GTP-bound form, and their internal GTPase

activity resets them to the inactive, GDP-bound ("off") form. Two members of this family have received substantial experimental attention and serve as representative models for protein-nucleotide interactions. Elongation factor Tu is responsible for transporting and coupling aminoacyl tRNA to the bacterial ribosome where the amino acid is added to the nascent peptide chain. EF-Tu-GTP binds with high affinity to aa-tRNA and ribosomes, both of which increase the internal GTPase activity of the protein, yielding EF-Tu-GDP, which dissociates from the ribosome. p21 proteins are products of the proto-oncogenic *ras* genes that are found in many mammalian cells. Their point-mutated forms are prevalent in many human tumors (Barbacid, 1987). Although little is known regarding the biochemical nature of p21 action, it is generally believed that p21-GTP exerts a signal that somehow induces cell division or proliferation (Hall, 1990; Grand & Owen, 1991). The internal GTPase activity associated with p21 is enhanced by external factors (GAP; Trahey & McCormick, 1987), yielding a non-signaling, p21-GDP product. The point-mutated oncogenic p21 proteins are remarkably conserved: they are altered in specific residues involved with nu-

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Abbreviations: p21, gene product of the human c-H-*ras* proto-oncogene; EF-Tu, elongation factor Tu from *Escherichia coli*; aa-tRNA, aminoacyl tRNA; GDP, guanosine 5' diphosphate; IDP, inosine 5' diphosphate; 6-thio-GDP, 6-thioguanosine 5' diphosphate; 6H-GDP, 6-hydroguanosine 5' diphosphate; GMP-PCP, guanylyl (β , γ -methylene)-diphosphate; Tris, tris(hydroxymethyl) aminoethane; DTE, 1,4-dithioerythritol; PMSF, phenylmethylsulfonyl fluoride; EDTA, (ethylenedinitrilo)-tetraacetic acid; RP-HPLC, reverse-phase HPLC; DTT, dithiothreitol; TEAB, triethyl-ammonium bicarbonate.

cleotide interactions and generally exhibit substantially lower GTPase activity and altered nucleotide binding characteristics.

Vibrational spectroscopy has several attributes that make it a suitable, and sometimes ideal, tool for probing protein–ligand interactions. The vibrational properties of a bound ligand are probably the most sensitive indication of the nature and extent of its interactions with the protein. Vibrational spectroscopy directly probes chemical bonds and such parameters as bond order, length, and symmetry (cf. Carey, 1982). An important outcome of the present study is a demonstration that hydrogen bonds between a ligand and residues in its protein binding site may be quantitatively studied. Furthermore, the method is non-destructive, and the reporter group is the molecule under study itself. An experimental constraint of vibrational spectroscopy is that the background signal from the protein itself overwhelms the ligand signal. This has been largely overcome in recent years by using Raman difference spectroscopy (Yue et al., 1984, 1989; Callender et al., 1989; Deng et al., 1989a, 1989b, 1989c, 1991, 1992). Because G-proteins are unstable in the absence of a bound nucleotide, we have adopted a slightly different strategy to determine the Raman spectra of bound nucleotide. A difference Raman spectrum is formed between a nucleotide and its derivative labeled with a stable isotope at a selected position (“isotope editing” spectroscopy; Manor et al., 1991). Because the two molecules are chemically identical, this can be performed with the nucleotides bound to the protein. Thus, GDP specifically deuterated at the guanine 8 position was incorporated into the binding site of EF-Tu and p21, thereby tagging the vibrational modes associated with the labeled atom. This enabled us to confirm the *in situ* nucleotide conformation as well as the interactions (or lack thereof) near the C8 position of the guanine ring (Manor et al., 1991).

In this report we investigate interactions between GDP (and analogs) and EF-Tu and p21 for two other positions within the purine base: the 6-keto and 2-amino positions. Both have been suggested by X-ray diffraction studies to be directly involved with specific binding interactions between the protein and the nucleotide. Hydrogen bonding between either of these “handles” and the protein is easily observed and characterized in our study here and then compared to known biochemical and structural data. The magnitudes of the observed spectral shifts reveal biochemically important differences between the two proteins that may be unexpected from the known structural similarities of their binding pockets.

Results

Our approach toward the measurement of the Raman spectra of ligands bound to proteins has been to perform classical Raman difference spectroscopy. The spectrum of protein·ligand complex is measured, as is the spectrum of the apoprotein. The difference spectrum is determined, and this contains mostly bands that are due to the bound ligand (e.g., Yue et al., 1989). Because the intensities of the bands associated with the bound ligand are on the order of a few percent or less smaller than those for the protein, a very sensitive difference spectrometer is required to enable the subtraction of the apoprotein spectrum. Ours is now able to accurately measure differences of 0.1% or less. In general, G proteins are very unstable in the absence of bound nucleotides. Thus, probing the vibrational properties of the bound nucleotide by subtracting the Raman spectrum of the

apoprotein from that of the protein·nucleotide complex is not a viable option. In a previous paper (Manor et al. 1991), we devised an alternative strategy for measurements of nucleotides bound to the p21 and EF-Tu G-proteins, which we called “isotope editing.” The difference spectrum is formed between two protein·nucleotide complexes where, in one of the complexes, the nucleotide is labeled with a stable isotope. This induces spectral shifts in the vibrational modes associated with motions of this labeled atom. Thus, all Raman bands, including those of the apoprotein and those of the bound nucleotide that *do not* involve the isotopic tag, cancel out in the difference spectrum. In that study, we were unable to measure the frequencies of modes that characterize two previously proposed binding “handles” between the apoprotein and the nucleotide, the guanine ring carbonyl at position 6 and the 2-amino group. Both of these positions are implied from X-ray diffraction studies to be crucial for *in situ* anchoring and specificity of GDP for the binding site via hydrogen bonding to protein residues (Jurnak, 1985; Pai et al., 1990; Kjeldgaard & Nyborg, 1992).

Here, we extend our previous studies with the additional isotopic label 6-¹⁸O-GDP. Also, we use an alternative route that “tags” specific vibrational coordinates by *chemical* modification at the selected positions; this approach is suitable for instances where isotopic labeling is either more complicated or more expensive from a synthetic point of view. As will be seen below, the difference spectrum formed by subtracting the spectrum of protein·nucleotide from that of protein·nucleotide analog offers a satisfactory spectroscopic solution to the problem of subtracting the background apoprotein spectrum. Accordingly, two guanine nucleotide analogs were chosen: IDP, which lacks GDP’s 2-amino group, and 6-thio-GDP, which contains a sulfur atom replacing the 6-oxygen. The chemical structures of these nucleotides are shown in Figure 1.

Nucleotide spectroscopy

The vibrational spectra of guanine and hypoxanthine as well as their respective nucleosides and nucleotides have been published

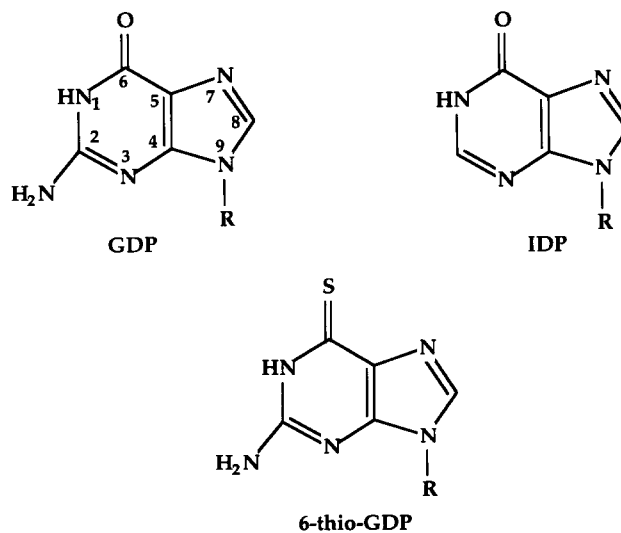


Fig. 1. Chemical structures of nucleotides used in this study. R = ribose 5' diphosphate.

and discussed previously (Psoda & Shugar, 1971; Lane & Thomas, 1979; Ferreira & Thomas, 1981; Majoube, 1985; Sheina et al., 1987; Lagant et al., 1991). However, we show solution data of GDP and IDP for reference as well as those for 6-thio-GDP in Figure 2, and we follow the previous spectral assignments in our discussion below. When IDP and GDP are measured under identical conditions in our difference spectrometer, a difference Raman spectrum such as that shown in Figure 3A is obtained. Because the spectra of both GDP and IDP are dominated by bands that are associated with the purine base moiety of the molecule (guanine and hypoxanthine, respectively), the major negative and positive peaks in Figure 3A arise from this part of the molecule. Since the only chemical difference between the two molecules is the presence of the 2-amino group in GDP, this difference spectrum exemplifies the different types of effects a single substitution has on the vibrations of a ring system. For example, GDP delocalized purine breathing motions at $1,577\text{ cm}^{-1}$, assigned primarily to the N3-C4 and C4-N9 stretching motions of the ring, and at $1,487\text{ cm}^{-1}$, assigned primarily to N7=C8 and C4=C5 stretching, are shifted to $1,554$ and $1,473\text{ cm}^{-1}$, respectively, in IDP. Localized motions that are directly associated with the "extra" amino group present in GDP are also clearly affected. These include the external C-N stretching mode observed at $1,178\text{ cm}^{-1}$ and the $-\text{NH}_2$ scissoring mode at $1,646\text{ cm}^{-1}$ in the GDP spectrum (Tajmir-Riahi & Theophamides, 1984), both of which are absent from the IDP spectrum. Importantly for this study, the somewhat weak and broad carbonyl (C=O) stretching mode, located at $1,680\text{ cm}^{-1}$ in GDP, shifts and becomes stronger and sharper at $1,692\text{ cm}^{-1}$ in the IDP spectrum (Fig. 2B). In the difference spectrum of Figure 3A, IDP's carbonyl peak clearly shows up as a negative peak at $1,695\text{ cm}^{-1}$. On the other hand,

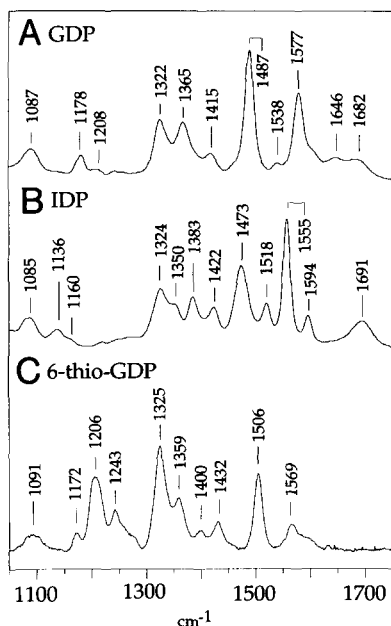


Fig. 2. Solution Raman spectra of (A) GDP, (B) IDP, and (C) 6-thio-GDP. Samples are at 0.1 M in H_2O , $\text{pH } 7.5$. Excitation is with 150 mW of the 488-nm Ar^+ laser line for A and B, and 150 mW of 568.2-nm line from a Kr^+ ion laser for C. In each case, the Raman spectrum of solvent was collected and subtracted out, and the resultant difference spectrum is shown.

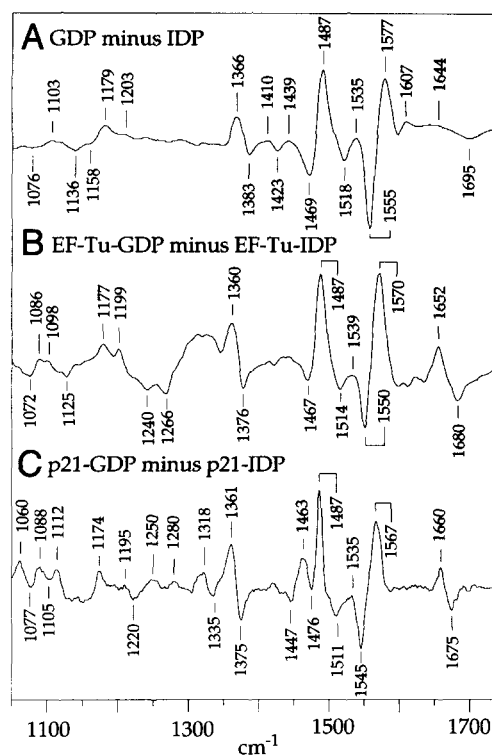


Fig. 3. Raman difference spectra formed between GDP and IDP (A) in solution, (B) complexed with EF-Tu, and (C) complexed with c-H-ras p21. **A:** Difference spectrum obtained from the data in Figure 2A and B. **B:** EF-Tu is at ca. 2.5 mM in buffer R and excitation is with the 530-nm line from a Kr^+ ion laser. **C:** p21 is at ca. 3.5 mM in buffer R and is excited with the 568.2-nm line from a Kr^+ ion laser.

GDP's weaker and broader carbonyl peak is essentially unobserved in the solution difference spectrum. The solution spectrum of 6-thio-GDP is shown in Figure 2C. The replacement of GDP's keto oxygen by sulfur affects a number of GDP's bands. We will not analyze this spectrum here except to point out that GDP's carbonyl, C=O, stretch found at $1,682\text{ cm}^{-1}$ and its $-\text{NH}_2$ scissors mode at $1,647\text{ cm}^{-1}$ are absent in the 6-thio-GDP spectrum (since C=S bond vibrations occur at much lower frequencies than C=O [Bellamy, 1980; Colthup et al., 1990]). Moreover, the 6-thio-GDP spectrum contains no bands in the $1620\text{--}1750\text{-cm}^{-1}$ region. Consequently, its difference spectra with GDP (Fig. 4D) and with IDP (Fig. 4F) show clearly GDP's and IDP's C=O stretch and GDP's $-\text{NH}_2$ scissoring modes.

Isotopic labeling of the guanine 6-position oxygen with ^{18}O shifts the carbonyl stretching mode to lower frequencies. As seen in Figure 5, this mode shifts from $1,685\text{ cm}^{-1}$ to $1,675\text{ cm}^{-1}$, similar to the results obtained from isotopic labeling of other atoms in this nucleotide (Manor et al., 1991).

Protein complex spectroscopy

Figure 3 shows difference spectra formed between GDP and IDP when bound to EF-Tu (Fig. 3B) and p21 (Fig. 3C). The solution difference spectrum is given in Figure 3A for comparison. An expanded view of the important high-frequency region is shown in Figure 4. Also shown in Figure 4 are the difference spectra between GDP and 6-thio-GDP in solution and in EF-Tu and

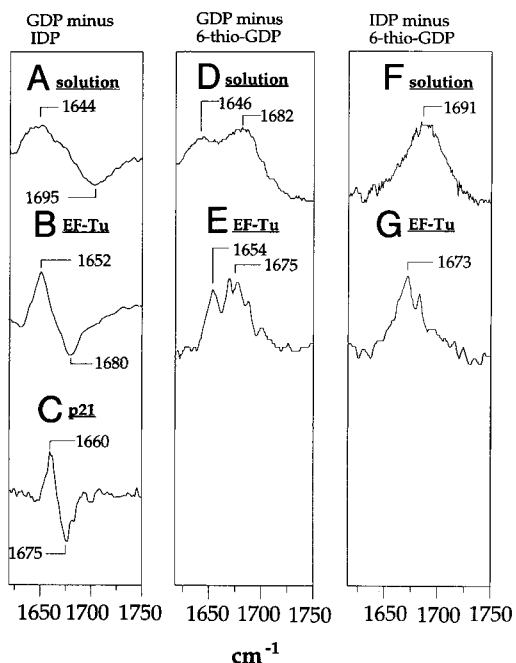


Fig. 4. Expanded view of the high-frequency region for the difference Raman spectra formed between the various nucleotides as noted on the top of each panel. The left-hand panel (GDP minus IDP) is copied from Figure 3. The middle and right-hand panels are for EF-Tu complexed with IDP and 6-thio-GDP prepared as described in the Materials and methods. Solution spectra (D, E) were obtained with the 568.2-nm line, and protein spectra were obtained with the 530.1-nm line from a Kr⁺ ion laser.

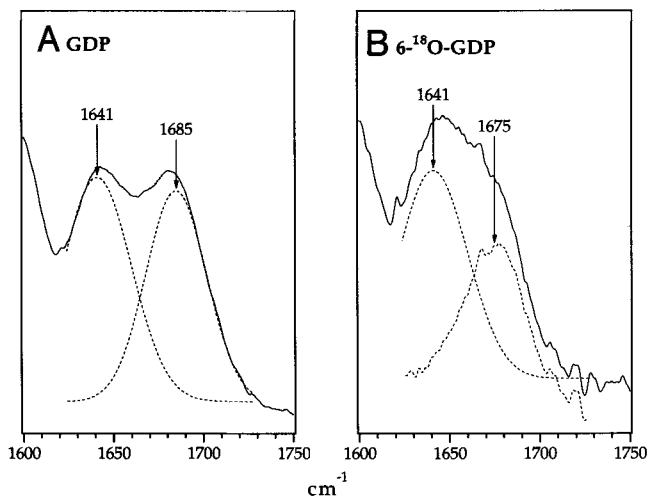


Fig. 5. An expanded view of the C=O stretching region of (A) GDP and (B) 6-¹⁸O-GDP. Samples were at 0.1 M in water, pH 7.4, and spectra were obtained with 180 mW of the 568.2-nm line from a Kr⁺ ion laser. Both spectra are normalized to the intensity of the 1,577-cm⁻¹ peak, which is not affected by the isotopic tag. **A:** Data are best fitted to the sum of two Lorentzian functions, shown as dashed lines. **B:** In order to find the exact position of the carbonyl stretching mode, which here strongly overlaps the -NH₂ scissoring mode (the 1,641-cm⁻¹ peak), the Lorentzian peak at 1,641 cm⁻¹, obtained from the fitting of the GDP spectrum (panel A), was subtracted, leaving the "isolated" C=O stretch at 1,675 cm⁻¹. Thus, this mode shifts down 10 cm⁻¹ upon isotopic labeling.

those between IDP and 6-thio-GDP in solution and in EF-Tu in the high-frequency region.

Protein difference spectra formed between protein·nucleotide and protein·nucleotide analog will contain nucleotide bands that shift in frequency or are absent in the spectrum of the analog. In addition, it is possible—even expected—that the difference spectra will also contain bands that arise from protein vibrations that are perturbed differently in their interaction with the nucleotide compared with the nucleotide analog; changes in the protein secondary structure composition will result in amide I intensity changes yielding spectral "signatures" similar to those observed here. We may safely assign some of the bands in the protein difference spectra as arising from the bound nucleotide for the following lines of evidence.⁴ The Raman cross sections of the aromatic purine rings and analogs are quite large compared to the vast majority of apoprotein vibrations and their expected changes (Deng et al., 1989c). For example, the intensity of the 1,487-cm⁻¹ band in the GDP spectrum is about 3% of that of the 1,449-cm⁻¹ protein band of EF-Tu and about 6% of the corresponding protein band in the p21 spectrum (Manor et al., 1991). From our previous studies, which determined difference spectra between EF-Tu and p21 containing isotopically labeled and unlabeled nucleotides (and hence the difference spectra could not include any apoprotein bands), this band's position and intensity are unaffected by binding to these two proteins. In the protein GDP minus IDP difference spectra of Figure 3B and C, an intense band is also observed at 1,487 cm⁻¹, at the solution position (Fig. 3A), and its intensity relative to apoprotein bands is preserved. Thus, it is certain that this band arises from the bound nucleotide. On the same grounds, we can assign other major bands in the protein difference spectra to nucleotide bands that preserve their relative intensity from the solution difference spectrum and that show either no or only small shifts upon binding. Those bands of special interest for this study that meet these criteria, their internal mode assignment, and shifts upon binding are tabulated in Table 1. This assignment of the two bands in the 1,600–1,700-cm⁻¹ range in the protein difference spectra to the nucleotide C=O stretch mode and the -NH₂ scissor mode is reinforced by observation of these bands in more than one difference spectrum. For example, the -NH₂ scissor mode is observed clearly at the same position and relative intensity in EF-Tu in the difference spectrum formed between GDP and IDP as in that formed between GDP and 6-thio-GDP. Moreover, no negative peaks are observed in the GDP minus 6-thio-GDP and IDP minus 6-thio-GDP protein difference spectra, as are observed when apoprotein peaks are affected upon ligand binding (Deng et al., 1989a, 1989b, 1989c).

The ¹⁸O-labeled nucleotide gives us confidence as to the assignments and shifts discussed above, and further shows that the

⁴ To further ascertain that the observed signals originate from changes in the nucleotide modes rather than from amide I changes, we repeated these experiments in D₂O-based buffers. However, the rates of backbone H → D exchange in the GDP complex are different from those in the IDP complexes, thereby producing difference signals at the amide region that mask the nucleotide-derived spectral differences. This is especially obvious when following the deuteration time course, as those differences get smaller and smaller upon long incubations in D₂O. Unfortunately, the time required for complete exchange in these proteins is on the order of days, during which denaturation occurs, as judged by both spectroscopic and functional criteria.

Table 1. Frequencies (in cm^{-1}) of selected nucleotide vibrations in solution and their frequency shifts when complexed to p21 and elongation factor Tu

Mode	ν (solution)	$\Delta\nu$ (EF-Tu)	$\Delta\nu$ (p21)
GDP vibrations			
N7=C8; C4=C5 stretch	1,487	0 ^{a,b}	0 ^{a,b}
N3-C4; C4-N9 stretch	1,577	-7 ^b	-10 ^b
C2-NH ₂ stretch	1,178	-2 ^b	-5 ^b
-NH ₂ scissor	1,646	+8 ^{b,c}	+16 ^b
C=O stretch	1,685	-15 ^{c,d}	-10 ^d
IDP vibrations			
C4=C5 stretch	1,555	-5 ^b	-10 ^b
C=O stretch	1,691	-15 ^c	-20 ^b

^a Shift in frequency obtained from Manor et al. (1991).

^b Difference spectrum formed between GDP and IDP.

^c Difference spectrum formed between GDP and 6-thio-GDP.

^d Difference spectrum formed between GDP and 6-¹⁸O-GDP.

^e Difference spectrum formed between IDP and 6-thio-GDP.

observed difference features are arising from nucleotide modes rather than conformational transitions in the protein. Figure 6 shows the difference spectrum formed between GDP and ¹⁸O-GDP in solution and when bound to the two proteins. Overall, the spectra are composed of sharp derivative-like features that result from frequency shifts of particular Raman bands. Some of these result from isotopic tagging of ring modes that are coupled to the 6-position oxygen. For example, GDP's band at 1,487 cm^{-1} , assigned to the N7=C8 and C4=C5 stretching modes, shifts downfield four wavenumbers, producing a derivative pair at 1,492 cm^{-1} and 1,478 cm^{-1} in the difference spectrum. This band was shown to undergo a similar shift upon deuteration of C8 (Manor et al., 1991) and virtually any other modification of the guanine ring (see above).

Discussion

The frequencies of the bands observed in vibrational spectroscopy are determined by the masses of the atoms and bond force constants of the internal coordinates that make up the observed normal mode. The force constants are functions of the distribution of the electrons in the internal coordinates. Thus, the observed band frequencies report on several structural attributes of the interacting atoms. In our previous work on the binding of GDP to EF-Tu and p21, we showed that the purine ring-ribose glycosidic bond adopted an anti- conformation while the pucker of the sugar is C2' *endo*- (Manor et al., 1991), in agreement with crystallographic studies (Jurnak, 1985; Pai et al., 1989; Kjeldgaard & Nyborg, 1992). Virtually all Raman bands in the difference spectra of the protein-bound nucleotides are sharper than those observed in solution, the effect being more pronounced with p21 than with EF-Tu. We interpret this qualitative difference to arise from a more constrained conformation of the nucleotide when bound to the protein. Consistently, the binding constant of GDP to both proteins is very high, and GDP binds significantly more tightly to p21 ($K_d = 2 \times 10^{-11}$ M [John et al., 1990]) than to EF-Tu ($K_d = 2 \times 10^{-9}$ M [Delaria & Jurnak, 1989]).

Our major interest in this paper centers on the hydrogen bonding interactions that exist between the bound nucleotide's pu-

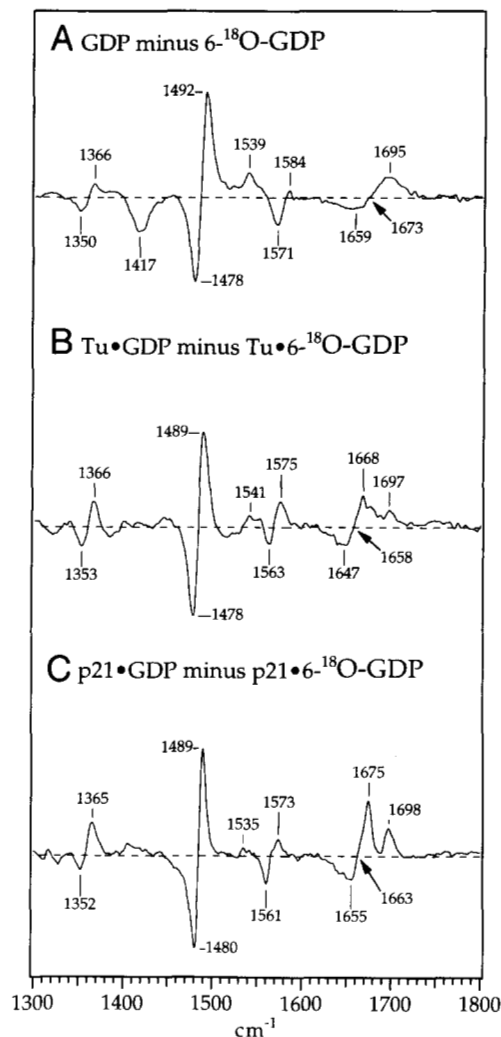


Fig. 6. Raman difference spectra of GDP minus 6-¹⁸O-GDP (A) in solution and when bound to (B) EF-Tu and (C) p21. The "zero crossing" points of the spectra are labeled with arrows. Conditions for A are as in Figure 5. EF-Tu and p21 are at ca. 2.5 mM and 3.5 mM, respectively, in buffer R. Raman scattering is excited with the 568.2-nm line from a Kr⁺ ion laser.

rine ring and EF-Tu and p21. For both proteins, high-resolution X-ray crystallographic structures have been determined, and a set of interactions between the guanine moiety of GDP and an array of conserved residues was implied (Jurnak, 1985; Pai et al., 1989; Kjeldgaard & Nyborg, 1992). Utilizing the conventional crystallographic criteria for hydrogen bonding (i.e., donor to acceptor distance ≤ 3.4 Å), the guanine base is anchored in the binding site with seven hydrogen bonds in EF-Tu and five hydrogen bonds in p21. However, it is not possible to assess the magnitude of these interactions with crystallographic data because of the limited accuracy of such studies, even for the 1.3-Å-resolution study now available for p21 (Pai et al., 1990; Kjeldgaard & Nyborg, 1992). The reason for this is that the shift in distance between the hydrogen bond donor and acceptor of only a few tenths of an Ångstrom results in a major change in the hydrogen bond energy. On the other hand, the electrons associated with the moiety forming the hydrogen bond respond quite strongly to this interaction, and this leads to easily measurable

changes in vibrational force constants and the associated frequencies of the observed bands. Thus, observed changes in the frequency of bands associated with moieties undergoing hydrogen bonding upon binding can be used as spectroscopic read-outs for the magnitude of the *local* binding interaction and changes in the interaction. In the following discussion we will assess the variation in the strength of the hydrogen bond by monitoring appropriate frequency shifts.

The intense purine peak that appears at $1,487\text{ cm}^{-1}$ for GDP and at $1,473\text{ cm}^{-1}$ for IDP in solution (see Fig. 2) is assigned to the ring $\text{N7}=\text{C8}$ and $\text{C4}=\text{C5}$ stretching motions coupled with 8-CH deformation (Majoube, 1984, 1985; Lagant et al., 1991), and the position of this band was shown to be sensitive to the magnitude of hydrogen bonding to N7 (Nishimura et al., 1986). When bound to both EF-Tu and p21, the position of this band remains unchanged from its solution value (Table 1), suggesting no change in interaction between N7 of the nucleotide and the binding site relative to its interaction with water. Lack of a change in interaction near the 7-position is supported also by previous Raman and chemical modification studies (Wittinghofer et al., 1977; Manor et al., 1991). These results do not imply that there are no interactions between N7 and its protein environment. There is presumably some hydrogen bonding with water for GDP in solution, and X-ray studies show N7 to be close enough to Asn 116 in p21 (3.2 \AA) and Asn 135 in EF-Tu (3.0 \AA) for effective hydrogen bonding. As for the $\text{C4}=\text{C5}$ coordinate, indeed no obvious interacting protein residue is seen in the X-ray structure of either protein·nucleotide complex.

A pronounced spectral shift is observed in the ring mode assigned primarily to the combined vibrations of $\text{N3}-\text{C4}$, $\text{N9}-\text{C4}$, and $\text{C2}-\text{N3}$ stretching motions (Majoube, 1985; Lagant et al., 1991). This band, observed at $1,577\text{ cm}^{-1}$ in GDP and at $1,555\text{ cm}^{-1}$ in IDP, shifts down by 7 cm^{-1} upon binding to EF-Tu and by 10 cm^{-1} when bound to p21 (see Table 1). This mode has shown to be sensitive to N3 labeling (Delabar & Guschlbauer, 1979) and to solvent polarity; its frequency is down-shifted by 12 cm^{-1} when guanosine is dissolved in DMSO and increases gradually with incremental additions of water (data not shown). The data therefore suggest that the protein-induced shifts of this mode arise primarily from the different polarity of the environment of the nucleotide when in water versus when bound to the protein. This is in accord with the immediate surroundings of this portion of the guanine base, as seen in the X-ray diffraction studies of the two proteins. The $\text{C2}-\text{N3}-\text{C4}-\text{N9}$ side is "sandwiched" inside a hydrophobic pocket formed by Phe 28 and Lys 117 in p21 and Leu 175 and Lys 136 in EF-Tu. Some contribution to the observed shift could also arise from coupling of this mode to the hydrogen bonded amino group (discussed below).

A major effect of binding is seen on the vibrational modes associated with positions 2 and 6 of the guanine base. In general, the formation of a stronger hydrogen bond will increase the frequency of a donor deformation vibration-like $-\text{NH}_2$ scissoring while decreasing the frequency of an acceptor stretching vibration such as the $\text{C}=\text{O}$ stretch (Vinogradov & Linnel, 1971; Joesten & Schaad, 1974; Deng et al., 1989a, 1991). This is because the $\text{H}-\text{N}-\text{H}$ scissoring motion will be "stiffened" by the hydrogen bond. On the other hand, electrons are drawn from the $\text{C}=\text{O}$ bond by attractive electrostatic interactions that lower its bond order and hence the frequency of the $\text{C}=\text{O}$ stretch. Modes that involve motion that is fairly localized to a small

group of atoms, like the keto stretch and the $-\text{NH}_2$ scissor, have been found to follow empirical relationships between the vibrational frequency of a group mode and interaction enthalpy between the group and a hydrogen bond donor/acceptor. These are called Badger–Bauer relationships, because these authors first established such a correlation in model compound studies (cf. Joesten & Schaad, 1974). Both experimental studies (Thijs & Zeegers-Huyskens, 1984a, 1984b) and recent theoretical calculations (Latajka & Scheiner, 1990) suggest that the frequency of a keto stretch follows a Badger–Bauer-like relationship in that the stretching frequency is directly proportional to the strength of the hydrogen bond enthalpy between the carbonyl and its nearby proton donor. There have been no direct measurements of the $-\text{NH}_2$ rocking mode in this regard, but it is reasonable, as a first-order approximation, to presume a Badger–Bauer-like relationship for this mode as well (cf. Deng et al., 1991). The susceptibility of a particular keto group to polarization by hydrogen bonding, and therefore the exact relationship between $\Delta\nu$ and ΔH , depends on the internal coordinates that make up the specific normal mode and, hence, the degree to which close local groups affect the electronic distribution of the $\text{C}=\text{O}$ moiety. In principle, it is possible to determine experimentally the magnitude of the hydrogen bond from calibration studies in solution with various donors/acceptors at different concentrations and temperatures. This has not yet been performed for guanine nucleotides due to their insolubility in the required inert solvents. In the discussion below, however, it should be kept in mind that the shifts in frequency are proportional to the strength of the interaction energy, and this forms a basis of comparing effects in EF-Tu with those in p21.

Accordingly, the amine deformation mode of GDP, observed at $1,646\text{ cm}^{-1}$ in solution, is shifted up by 8 and 16 cm^{-1} upon nucleotide binding to EF-Tu and p21, respectively, from Table 1 (consistently, the stretching frequency of GDP's exocyclic $\text{C}-\text{N}$ bond at $1,178\text{ cm}^{-1}$ shifts down 2 cm^{-1} and 5 cm^{-1} upon binding to the two proteins [see Manor et al., 1991]). The carbonyl stretching frequencies of both GDP and IDP follow qualitatively the same trend; the ketone is hydrogen bonded to the proteins more strongly than when in water, since the $\text{C}=\text{O}$ stretch shifts down 15 cm^{-1} for GDP in EF-Tu and down 10 cm^{-1} for GDP in p21. Taken together, these results indicate stronger hydrogen bonding (ΔH) of the 6-keto and 2-amino moieties of GDP to EF-Tu and p21 than to water, and are consistent with the available structural information on the binding site. Interactions with these positions are conserved in EF-Tu and p21 as well as among other members of the GTPase superfamily, as part of the NKXD motif (e.g., Kaziro et al., 1991). The 2-amino group of GDP is known to be only 3 \AA or less away from an invariant Asp residue (Asp 138 in EF-Tu and Asp 119 in p21), while the 6-keto group is hydrogen bonded to the main chain NH of a conserved alanine residue (Ala 146 in p21 and Ala 174 in EF-Tu).

Most hydrogen bond-induced shifts observed in the present study are such that the strength of the hydrogen bond is greater in p21 than in EF-Tu (see Table 1). This is consistent with the observation that p21 binds GDP (or IDP [Wittinghofer et al., 1977; Sigal et al., 1986]) some 100-fold more strongly than does EF-Tu. An interesting deviation from this generalization is the $\text{C}=\text{O}$ stretching mode of GDP; a 10-cm^{-1} shift is observed when the nucleotide is bound to p21, versus a 15-cm^{-1} shift in EF-Tu. Apparently, the stronger binding of GDP to p21 is me-

diated significantly via the external amino group. For IDP, lacking this moiety, the primary binding "handle" is the 6-keto group, the hydrogen bonding to which is much tighter for p21, possibly through a different conformational arrangement of the binding pocket. Verification of this awaits structural studies on the protein·IDP complexes (should such measurements be feasible, since a series of small protein changes, outside the resolution of X-ray studies, may well be responsible for the observed shifts in frequency).

The strengths of hydrogen bonds are thought to play a key role in determining the specificity of interaction between a protein and ligand, and these interactions are important to enzymatic catalysis. Despite their importance, there is very little known about the quantitative characteristics of these bonds (cf. Fersht et al., 1985). One lesson from the presented data is that a simple determination of the structure of the active site is not sufficient to determine hydrogen bonding strengths. While the binding sites for the 6-keto and 2-amino groups are said to be conserved between EF-Tu and p21 and are indeed very similar, the relative strengths (ΔH) of the hydrogen bonds formed with these groups and the two proteins are very different. This may be a completely unexpected result of the present study. Another lesson is that great care should be taken in inferring strengths of hydrogen bonding from competition binding experiments. This lesson results from the comparison of GDP to IDP binding. It might not be unreasonable to suppose that the difference in binding energy of the two ligands is solely due to the absence of the 2-amino group in IDP, because both EF-Tu and p21 bind GDP about 100-fold more strongly than IDP and because the binding pockets are very similar. The data show, however, that the 6-keto group binds more weakly in p21 relative to EF-Tu for GDP and just the *reverse* for IDP. Thus, the presence or absence of the amino group influences the strength of a protein–ligand interaction removed from it, and the binding affinity would appear to result from a number of interactions in a complex way. For these two G proteins, this result is perhaps not so unexpected on second thought. Both proteins very strongly bind purine-based nucleotides and, in fact, are unstable without them, suggesting that their structure is very much dependent on the presence of the nucleotide.

Materials and methods

Chemicals

GDP, GMP·PCP, IDP, and 6-thioguanosine were obtained from Sigma or Boehringer Mannheim. (–)-2-Amino-6-chloropurine riboside came from Aldrich. $H_2^{18}O$ (98%) was from Isotec Inc., and 3H -GDP (12 Ci/mmol) came from Amersham. All other chemicals were of the highest purity grade available. 6-Thio-GDP was synthesized from 6-thioguanosine using published procedures (Eccleston & Trentham, 1977; Breter & Mertes, 1990).

Proteins

EF-Tu-GDP and *c-H-ras*-p21-GDP were purified and prepared for spectroscopy as described previously (Manor et al., 1991). Purified proteins were stored at $-60^\circ C$ in buffer A (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.5 mM DTE, 1 mM NaN_3 , 0.1 mM PMSF) containing 0.1 mM GDP, and were routinely checked for activity using a radioactive GDP binding assay (Miller & Weissbach, 1974).

6- ^{18}O -GDP synthesis

6- ^{18}O -labeled guanosine was prepared by mixing (–)-2-amino-6-chloropurine riboside (80 mg) with 2 mL of 1 N $Na^{18}OH$ in $H_2^{18}O$. The mixture was refluxed for 5 h and neutralized with HCl gas. The precipitate was filtered and dissolved in 1 N HCl, filtered, and neutralized with NaOH. The white precipitate was filtered off, washed three times with cold water, and dried under reduced pressure at $110^\circ C$. The 6- ^{18}O -guanosine thus obtained was converted to 6- ^{18}O -guanosine monophosphate by published methods (Sowa & Ouchi, 1975).

To obtain the labeled diphosphate derivative, 6- ^{18}O -GMP (0.05 mmol) was mixed with ATP (0.21 mmol) in 6 mL of 50 mM Tris, 50 mM $MgCl_2$, pH 7.5, followed by addition of 0.5 units guanylate kinase. The mixture was incubated for 30 min at $30^\circ C$, then for 18 h at $5^\circ C$, evaporated to dryness, dissolved in 2 mL 0.1 M triethylammonium bicarbonate, and applied to a column of DEAE-Sephadex A-25 (2.5×40 cm; bicarbonate form). The column was developed with a linear gradient of 0.1–0.7 M triethylammonium bicarbonate (2 L each). The final yield of pure product was 80%. Purity was verified by RP-HPLC.

Preparation of IDP and 6-thio-GDP protein complexes

These were prepared in two stages: first, protein was complexed with GMP·PCP in the presence of alkaline phosphatase (agarose or acrylic beads linked; Sigma Chemical Co., Saint Louis, Missouri) as previously described (John et al., 1990). When all of the GDP had been converted (after ca. 2 h for EF-Tu and 1 h for p21; monitored by RP-HPLC), alkaline phosphatase was removed by filtration. EF-Tu-GMP·PCP complex obtained this way was incubated with 10-fold molar excess of IDP or 6-thio-GDP in buffer I (0.2 M Tris-HCl, 0.2 M $(NH_4)_2SO_4$, 5 mM DTT, 0.5 mM NaN_3 , pH 7.4) at $37^\circ C$ for 15 min. The mixture was then washed in a Centricon centrifugal concentrator (model 30 for EF-Tu and model 10 for p21, Amicon Corp., Beverly, Massachusetts) with buffer R (20 mM sodium phosphate, 10 mM $MgCl_2$, pH 7.4). p21-GMP·PCP obtained after the alkaline phosphatase reaction was passed through a PD-10-G25 column (Pharmacia-LKB, Piscataway, New Jersey) to remove excess GMP·PCP in solution. The protein then was incubated for 40 min at $25^\circ C$ with 30-fold molar excess of IDP in buffer I, and then washed with buffer R. The last step was repeated three times, after which the content of p21-IDP complex as determined by RP-HPLC was $>90\%$. Protein complexes of 6- ^{18}O -GDP were prepared as described earlier for 8D-GDP (Manor et al., 1991).

Spectroscopy

Raman spectra were measured with a Triplemate spectrometer (Spex Industries, Metuchen, New Jersey) coupled to either an optical multichannel array (OMA) or a charge coupled device (CCD) detector. The OMA system consists of a solid-state detector system (model DIDA-1000 water-cooled photodiode array with a model ST-100 detector controller; Princeton Instruments, Trenton, New Jersey). The CCD detector (model LN/CCD-1152UV with a model ST-135 CCD controller; Princeton Instruments) is cooled to $-100^\circ C$. Raman spectra were acquired, stored, and analyzed on a Macintosh IIx computer (Apple, Cupertino, California). A detailed description of the Raman difference spectrometer has been provided previously (Deng et al.,

1989c; Yue et al., 1989). For excitation, 150 mW of a selected laser line (indicated in the figure legends) from either an INOVA 2000 Ar⁺ ion laser or a CR-2000 Kr⁺ ion laser (Coherent Radiation Inc., Palo Alto, California) was used. Spectral lines were calibrated against known Raman lines of toluene and are accurate to within $\pm 2 \text{ cm}^{-1}$. Spectral resolution is $\pm 6 \text{ cm}^{-1}$.

Acknowledgments

This work was supported in part by grant MCB-8912322 from the National Science Foundation and grants GM 35183 and EY 03142 from the National Institutes of Health.

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