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Random-coil chemical shifts of phosphorylated amino acids

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Abstract

The ^{1}H, ^{13}C, ^{15}N and ^{31}P random-coil chemical shifts and phosphate pK_a values of the phosphorylated amino acids pSer, pThr and pTyr in the protected peptide Ac-Gly-Gly-X-Gly-Gly-NH_2 have been obtained in water at 25 °C over the pH range 2 to 9. Analysis of ROESY spectra indicates that the peptides are unstructured. Phosphorylation induces changes in random-coil chemical shifts, some of which are comparable to those caused by secondary structure formation, and are therefore significant in structural analyses based on the chemical shift.

Abbreviations: DQF-COSY: double-quantum filtered correlation spectroscopy; DSS: 2,2-dimethyl-2-silapentane-5-sulfonic acid; HMBC: heteronuclear multiple-bond correlation spectroscopy; HSQC: heteronuclear single-quantum correlation spectroscopy; ROESY: rotating frame Overhauser enhancement spectroscopy; TMP: trimethylphosphate.

Introduction

Random-coil chemical shifts are essential for interpreting chemical shifts in terms of structure formation and for theoretical approaches to chemical shift calculations (Wishart and Sykes, 1994; Case, 1998; Clore and Gronenborn, 1998; Wishart and Nip, 1998). Application of the chemical shift can be extended to analyzing structural changes induced by posttranslational modifications. An important reversible covalent protein modification is phosphorylation, which modulates protein function in a diverse range of cellular processes (Hunter, 1995; Johnson et al., 1998).

^{1}H and ^{31}P chemical shifts for the phosphorylated amino acids pThr, pTyr and pSer have been reported in the context GGXA, in which the N- and C-termini were unblocked (Hoffmann et al., 1994). However, given the differences in 'random-coil' chemical shifts measured in different peptide contexts (Merutka et al., 1995; Wishart et al., 1995a; Wishart and Nip, 1998) and the lack of ^{13}C and ^{15}N chemical shift data for phosphorylated amino acids, we have measured 'random-coil' ^{1}H, ^{13}C, ^{15}N and ^{31}P chemical shifts for phosphorylated pSer, pThr and pTyr in the context Ac-GGXGG-NH_2. These chemical shift and pK_a values provide a basis for understanding structural and functional changes due to protein phosphorylation.

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Methods

Peptides were synthesized using Fmoc chemistry in the CSU Macromolecular Resources Facility. The N- and C-termini were chemically blocked by acetylation and amidation to preclude end-charge effects. The identity of the peptides was confirmed with MALDI mass spectrometry; a single m/z peak corresponding to the molecular mass within 0.4 Da of the expected value was observed for each peptide.

NMR spectroscopy was performed with a Varian Unity Inova operating at 500.1 MHz for ^{1}H. Data were processed and analyzed using VNMR (Varian Instruments), NMRPipe (Delaglio et al., 1995) and NMRView (Johnson and Blevins, 1994). All data were acquired at 25 °C on peptides dissolved in 90%

H_2O/10% D_2O. pH was adjusted with HCl and NaOH, and found to vary by less than 0.05 pH units before and after each experiment. ^1H, ^{13}C and ^{15}N spectra were referenced to internal DSS (Wishart et al., 1995b). ^{31}P spectra were referenced to internal TMP at 0 ppm (Markley et al., 1998), which resonates 3.77 ppm downfield of phosphoric acid (Wilmad WGP-39).

Assignments were made independently of previously determined values of random-coil chemical shifts. ^1H assignments were based on spin system identification using DQF-COSY spectra and $d_{\alpha N}(i,i+1)$ connectivities in ROESY spectra (Derome and Williamson, 1990; Hwang and Shaka, 1992). ^{15}N and ^{13}C assignments were made using ^1H correlations in sensitivity-enhanced gradient HSQC (Palmer et al., 1991; Kay et al., 1992) and HMBC spectra (Bax and Summers, 1986). ^1H, ^{13}C and ^{31}P chemical shifts were measured from one-dimensional spectra. ^{15}N chemical shifts were measured from HSQC spectra.

pK_a values were determined from a least-squares fit of the chemical shift δ as a function of pH to

$$\delta = [\delta^{2-}(10^{pH-pKa}) + \delta^{-}]/[1 + 10^{pH-pKa}] \quad (1)$$

where δ^{2-} and δ^{-} are the chemical shifts of the dianionic and monoanionic forms of the phosphate group, respectively (cf. Cohen et al., 1970). Chemical shift changes reflecting the protonation step of the monoanionic phosphate group were not observed in the pH range studied (2–9), and so were not included as a fitting parameter.

Changes in ^1H chemical shift upon phosphorylation were calculated using random-coil values measured at pH 5 in the contexts Ac-GGXGG-NH_2 (Plaxco et al., 1997) and GGXGG (Merutka et al., 1995). The chemical shifts of GGXGG were measured at pH 5 and 5 °C, and so were corrected for temperature as described by Merutka et al. (1995). Changes in ^{13}C chemical shift upon phosphorylation at pH 3 were calculated using the random-coil chemical shifts measured in GGXGG at pH 2–3.5 and 25 °C (Thanabal et al., 1994). The ^{13}C data of Thanabal et al. were adjusted by +0.12 ppm to account for differences in referencing (Wishart et al., 1995b).

Results and discussion

Structural characterization of the phosphopeptides

For each peptide, strong $d_{\alpha N}(i,i+1)$ connectivities were the only interresidue NOEs observed in ROESY spectra (Figure 1). This result indicates that the peptides populate the β (extended) region of ϕ/ψ space, typical of unfolded peptides (Dyson and Wright, 1991), and that stable side-chain–side-chain interactions are not present. The NOE data strongly suggest that the peptides are unstructured, in accord with NMR studies of closely related oligopeptides (Merutka et al., 1995; Plaxco et al., 1997).

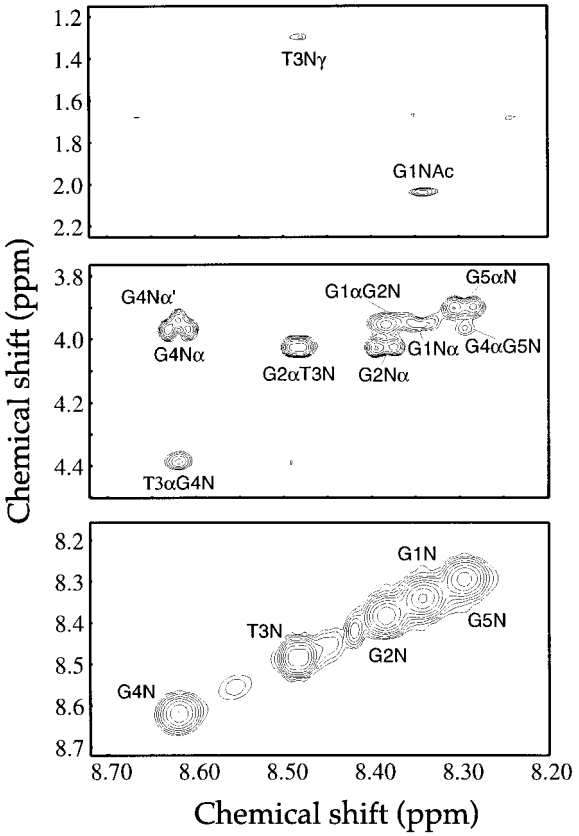

Figure 1. ROESY spectrum of Ac-GGpTGG-NH_2 (200 ms mixing time). $d_{\alpha N}(i,i+1)$ and certain intraresidue connectivities are observed unambiguously. In contrast, $d_{NN}(i,i+1)$ and side-chain–side-chain NOE connectivities are not observed. The H^N chemical-shift dispersion is sufficient for the unambiguous observation of $d_{NN}(i,i+1)$ NOE connectivities if present, with the exception of the $d_{NN}(1,2)$ connectivity.

Random-coil chemical shifts of pSer, pThr, and pTyr

Chemical shifts were measured over the pH range 2–9 and fit to Equation 1 (Table 1). Chemical shifts can be calculated with Equation 1 at any desired pH using the limits δ^{-} and δ^{2-} and pK_a values listed in Table 1.

^1H chemical shifts of pSer, pThr and pTyr have been previously measured in unblocked GGXA and fit to four pK_a values (Hoffmann et al., 1994). The fitting equation was not reported, and so we compare only the low pH ^1H chemical shift limits measured in GGXA

Table 1. Chemical shift and pK_a data

Resonance		δ_1	pK_a	δ_2	$\Delta\delta$ (ppm)		
					GGpXA	Ac-GGXGG-NH_2	GGXGG
pSer	P	1.16	5.96	5.06			
	N	115.5	6.02	118.3			
	H^N	8.65	6.03	9.29	−0.02	0.31	0.17
	H^α	4.60	6.00	4.43	0.03	0.10	0.09
	H^β	4.22	6.07	4.07	0.05	0.23	0.19
	H^β	4.13	*	4.07	−0.01	0.29	0.25
	CO	174.9	5.99	175.9			
	C^α	57.40	*	58.46			−1.08
	C^β	66.81	6.04	65.65			2.92
pThr	P	0.22	6.30	4.51			
	N	113.4	6.35	119.5			
	H^N	8.43	6.34	9.21	−0.05	0.26	0.12
	H^α	4.43	6.33	4.18	0.13	0.07	0.03
	H^β	4.66	6.33	4.41	0.17	0.33	0.33
	H^γ	1.33	6.34	1.27	−0.03	0.11	0.10
	CO	175.1	6.33	175.9			
	C^α	61.77	6.35	63.57			−0.28
	C^β	74.02	6.31	71.66			4.22
	C^γ	20.69	6.30	20.98			−0.88
pTyr	P	−3.07	5.96	0.95			
	N	120.1	*	120.3			
	H^N	8.21	*	8.23	−0.04	0.01	−0.16
	H^α	4.61	*	4.59	−0.02	0.09	0.05
	H^β	3.15	*	3.12	0.03	0.04	0.04
	H^β	3.02	*	3.01	0.04	0.04	0.09
	H^δ	7.22	6.03	7.17	0.00	0.07	0.07
	H^ϵ	7.14	*	7.13	0.02	0.27	0.29
	CO	176.7	*	176.7			
	C^α	57.04	*	57.04			−1.18
	C^β	38.73	*	38.70			−0.18
	C^δ	133.02	5.99	132.61			−1.58
	C^ϵ	123.19	*	123.19			3.72

^1H, ^{13}C, ^{15}N and ^{31}P chemical shifts are accurate to ±0.01, ±0.03, ±0.3 and ±0.04 ppm, respectively, except for the CO shift of pTyr which is accurate to ±0.1 ppm. δ^- and δ^{2-} are the chemical shift limits at low and high pH, respectively (see Equation 1). * denotes resonances for which chemical-shift changes with pH were too small for a reliable pK_a measurement. $\Delta\delta$ denotes differences in chemical shift upon phosphorylation. A positive change corresponds to a downfield shift.

with those measured here at pH 3. Most ^1H chemical shifts measured for the corresponding peptides are similar, with nonsystematic differences of 0.05 ppm or less (Table 1). Larger differences of 0.13 and 0.17 ppm are observed for the H^α and H^β chemical shifts of pThr, respectively (Table 1). Such differences in chemical shift may reflect sequence effects and chemical blocking of the N- and C-termini (Merutka et al., 1995; Wishart et al., 1995a).

Phosphate pK_a values

pK_a values for the equilibrium between the singly and doubly charged phosphate group of 5.96±0.09, 6.30±0.07 and 5.96±0.04 for pSer, pThr and pTyr, respectively, were obtained from the changes in the ^{31}P phosphate chemical shift (Figure 2). Similar values were obtained from the titration behavior of the ^1H and ^{13}C resonances (Table 1). The pK_a values measured

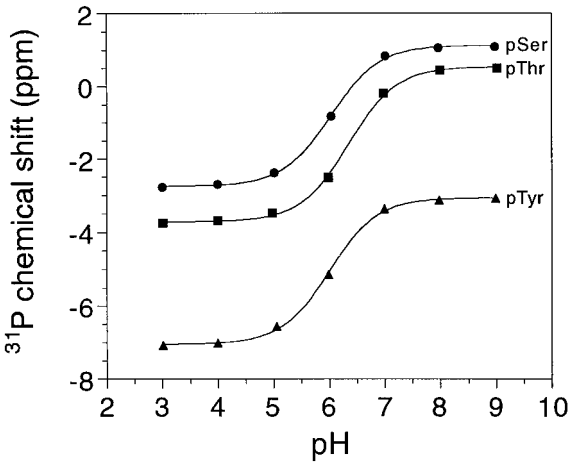

Figure 2. Changes in the ^{31}P chemical shift of the phosphate group with pH. The curves are least-squares fits to Equation 1 of the text.

here for pSer and pTyr, but not for pThr, are similar to values measured in GGXA (Hoffmann et al., 1994).

Changes in chemical shift upon phosphorylation

^{1}H chemical shifts for unmodified Ser, Thr and Tyr have been measured previously in several peptide contexts (Wishart and Nip, 1998). ^{1}H chemical shifts in GGXGG (Merutka et al., 1995) and Ac-GGXGG-NH_2 (Plaxo et al., 1997), and ^{13}C chemical shifts in GGXGG (Thanabal et al., 1994), provide the closest sequence contexts to that used here for comparing chemical shifts in unmodified and phosphorylated amino acids.

Significant, nonsystematic differences in ^{1}H (up to 0.33 ppm) and ^{13}C chemical shifts (up to 4.2 ppm) are observed upon phosphorylation (Table 1). The chemical shifts were measured under essentially equivalent conditions or corrected for temperature or referencing methods, and so are not simply due to chemical-shift referencing, pH or temperature. Several of the observed changes are significant in terms of characterizing structure, since these phosphorylation-induced changes are comparable to those induced by secondary structure formation (Wishart and Sykes, 1994).

Conclusions

We have characterized the effects of phosphorylation on the ^{1}H, ^{13}C, ^{15}N, and ^{31}P chemical shifts of the amino acids Ser, Thr and Tyr in the context Ac-GGXGG-NH_2. These chemical shifts and phosphate pK_a's provide a basis for interpreting NMR spectral changes due to protein phosphorylation, and for the application of chemical-shift methods for structural analysis.

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