Phosphorylation induces subtle structural changes in SpoIIAA, a key regulator of sporulation

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The phosphorylation state of SpoIIAA is a key factor in the regulation of sporulation in Bacillus subtilis. Previous crystallographic studies had led to the conclusion that phosphorylation alters the binding affinity of SpoIIAA for its partner proteins solely through the additional charge and bulk of the phosphoyl group: small structural changes observed elsewhere in the protein were considered to be random fluctuations rather than the result of phosphorylation. The results presented in the present paper show that NMR studies detect the same subtle structural changes in solution as those seen in the crystal, strongly implying that they are the direct result of phosphorylation. These subtle structural changes are similar to those that occur in a non-phosphorylated mutant that is defective in binding to one of its partner proteins. We propose that the structural changes which occur in SpoIIAA on phosphorylation act in concert with the phosphoyl group to alter its binding properties.

Key words: NMR, phosphorylation, SpoIIAA, sporulation, structural changes.

INTRODUCTION

Spores are produced by the soil bacterium Bacillus subtilis in response to nutrient deprivation. Early in sporulation, the cell divides asymmetrically to form two unequal compartments, the mother cell and the prespore. Differential gene expression occurs in these two compartments through the activation of specific transcription factors known as sigma factors. The prespore-specific sigma factor, σF, is the first of these to be activated. The three proteins that regulate σF are SpoIIA, SpoIIB and SpoIIE. SpoIAB is an anti-sigma factor, which can either bind to and inhibit σF, or act as a specific kinase for SpoIIAA by transferring the γ-phosphate of ATP to Ser58 of SpoIIAA. Before asymmetric septation, SpoIIAA is in a phosphorylated state, which, under physiological conditions, does not interact with SpoIIAB, leaving the latter free to inhibit σF. However, at the time of asymmetric septation, SpoIIE, the specific phosphatase for SpoIIAA-phosphate, becomes active in the prespore. The dephosphorylation of SpoIIAA allows it to interact with SpoIIAB, thereby releasing σF activity (reviewed by Kroos et al. [1]).

Phosphorylation is known to induce a variety of effects on protein structure (reviewed by Johnson and Lewis [2]). In some cases, no change in the protein conformation is detected: an example is isocitrate dehydrogenase, where the phosphoyl group itself acts as a steric and electrostatic block to the catalytic site. In contrast, some proteins, such as glycogen phosphorylase, undergo large-scale conformational changes on phosphorylation. For most other proteins, the effects of phosphorylation lie within these two extremes.

The NMR structure of the non-phosphorylated form of SpoIIAA from B. subtilis was determined in this laboratory several years ago [3]. Recently, Seavers et al. [4] obtained crystal structures for SpoIIAA, in both its phosphorylated and unphosphorylated forms, from the closely related species Bacillus sphaericus. The folding of these structures was largely similar to that previously determined by NMR. Although minor structural differences were observed between the two forms in a region near to the phosphorylation site, the data suggested this region to be inherently flexible. It was therefore concluded that there is no concerted movement of SpoIIAA on phosphorylation, and that changes in binding affinity of SpoIIAA for its partner proteins on phosphorylation are due entirely to the extra bulk and charge of the phosphoyl group. However, our previous 15N relaxation studies had indicated that the region in which structural movements are observed is not unusually flexible [5]. To investigate this subject further, we examined whether similar phosphorylation-induced structural changes occur in solution, by identifying induced chemical-shift perturbations in high-resolution NMR spectra. The influence of phosphorylation was also probed by monitoring changes in the pK₅ of histidine residues in the protein. In all these studies, unphosphorylated SpoIIAA was compared both with SpoIIAA-phosphate and with a mutant protein, SpoIIAAS58D (with a Ser58 → Asp mutation), which has similar biochemical properties to SpoIIAA-phosphate [6].

EXPERIMENTAL

Analytical ultracentrifugation (AUC)

Samples of phosphorylated and non-phosphorylated SpoIIAA proteins were overexpressed and purified as previously described in [7]. Proteins were used in the concentration range 7–125 μM, in a pH 7 buffer containing 25 mM KH₂PO₄, 75 mM NaCl and 1 mM dithiothreitol (DTT), which was also used as a blank in the AUC experiments. Molecular mass measurements were carried out using a Beckman Instruments Optima XL-I, equipped with scanning interference optics. Analysis of sedimentation equilibrium data to obtain the weight-average molecular mass was performed with Beckman software with Origin® 4.1, by fitting the Lamm equation.

Abbreviations used: AUC, analytical ultracentrifugation; DTT, dithiothreitol; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser effect; TROSY, transverse relaxation-optimized spectroscopy.

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Figure 1 Structural perturbations of SpoIIAA-phosphate from SpoIIAA

Superimposed on the NMR structure of SpoIIAA [3], significant chemical-shift perturbations (defined as > 0.1 ppm for hydrogen values or > 0.75 ppm for nitrogen values) are shown in dark grey. The absolute values of chemical-shift perturbations are available at http://www.BiochemJ.org/bj/372/bj3720113add.htm.

NMR analysis of backbone amides

Samples of uniformly 15N-labelled phosphorylated, non-phosphorylated and mutant SpoIIAA proteins were overexpressed and purified as previously described in [3,7], except that partial deuteration was achieved through the use of deuterium oxide as the sole solvent. Samples were prepared in a pH 6.8 buffer containing 25 mM K2HPO4, 25 mM NaCl, 1 mM DTT, 0.02% (w/v) NaN3 and 5% (v/v) 2H2O. Deuterated CHAPS (10 mM) was added to SpoIIAA-phosphate and SpoIIAAS58D to increase solubility. The addition of CHAPS did not alter the transverse relaxation-optimized spectroscopy (TROSY) spectrum of SpoIIAA-phosphate. The protein concentrations used were 0.5 mM for SpoIIAA-phosphate, 1 mM for SpoIIAA, and 0.5 and 1 mM for SpoIIAAS58D.

NMR experiments were performed at 750 MHz and at a temperature of 25 °C. Felix software was used for spectral processing and NMRView for spectral analysis. TROSY spectra (reviewed by Riek et al. [8]) were acquired for all three proteins and heteronuclear single quantum coherence (HSQC)-NOESY-HSQC spectra [9] acquired for SpoIIAA-phosphate and SpoIIAAS58D. Assignment was carried out by comparison with previous assignments for SpoIIAA [3] and verification that expected backbone amide nuclear Overhauser effects could be detected. Of the expected TROSY peaks, 99% were assigned for SpoIIAA, 96% for SpoIIAA-phosphate and 98% for SpoIIAAS58D.

pKₐ determination of histidine residues by NMR spectroscopy

Protein samples were overexpressed and purified as previously described in [3,7]. Samples were prepared at a protein concentration of 0.3 mM in buffer containing 25 mM K2HPO4, 25 mM NaCl, 1 mM DTT, 0.02% (w/v) NaN3 and a 2H2O concentration of greater than 95%. The pH titrations were carried out in ten steps for each protein over the pH range 5–8.5. The pH of the sample was adjusted by mixing samples of pH 5 and pH 8.5 and through the addition of 1 µl volumes of 0.1 M HCl and NaOH. One-dimensional proton NMR experiments were performed at 600 MHz and at a temperature of 25 °C. Felix software was used for spectral processing and analysis. Histidine C-H resonances were assigned by comparison of the spectra at pH 6.5 with previous assignments at this pH [3]. Origin 5.0 software was used to analyse the resulting NMR titration data. pKₐ and Hill coefficient values were obtained by fitting the data to a modified Hill equation as described by Markley [10].

RESULTS

NMR studies to compare unphosphorylated and phosphorylated SpoIIAA

Figure 1 shows that some parts of the protein experience significant chemical-shift perturbations when SpoIIAA is phosphorylated. The most substantially perturbed secondary-structure units are helix 2 and the loop preceding it, and helix 3 and its surrounding loops.

The spectra of SpoIIAA-phosphate have broader linewidths than those of SpoIIAA, indicating that, under NMR conditions, SpoIIAA-phosphate is effectively a higher molecular mass species. Molecular mass determination by AUC showed that, unlike unphosphorylated SpoIIAA, SpoIIAA-phosphate forms dimers in solution. SpoIIAA-phosphate was fully dimerized at concentrations even below those used in NMR experiments. At lower concentrations, including those which are known to occur under cellular conditions [11], SpoIIAA-phosphate is partially dimerized, though it is not known if this is significant in vivo. The chemical-shift perturbations detected in the NMR experiments with SpoIIAA-phosphate will therefore result both from the effects of dimerization and from any electrostatic and structural perturbations introduced by the phosphate. Fortunately, we were able to obtain a good approximation of the deconvolution of these two effects by using a mutant mimic of the phosphorylated form.

NMR studies to compare unphosphorylated SpoIIAA and SpoIIAAS58D

The SpoIIAAS58D mutant of SpoIIAA replaces the serine-phosphate with a negatively charged aspartate. Previous HPLC...
gel-permeation results suggested that SpoIIAAS58D forms dimers in solution, but less readily than SpoIIAA-phosphate [6]. This conclusion was borne out by examination of the TROSY spectra of SpoIIAAS58D, which showed that certain peaks in each spectrum were significantly reduced in intensity, and that this reduction in intensity was more striking in the spectrum at 1 mM than at 0.5 mM. This result suggests that, under NMR conditions, SpoIIAAS58D is in a ‘slow-exchange regime’ between its monomeric and dimeric forms. The less intense peaks correspond to resonances in the monomeric form that are altered on dimerization. At 1 mM, peaks which we take as corresponding to the dimeric form become clearly visible at similar positions to the monomeric form.
Crystallographic structures were prepared using co-ordinates which had been deposited with the European Bioinformatics Institute (EBI) with accession codes 1h4z (SpoIIAA) and 1h4x (SpoIIAA-phosphate). Ser57 of B. sphaericus SpoIIAA corresponds to Ser58 of B. subtilis SpoIIAA.

peaks observed only in the phosphorylated form of wild-type SpoIIAA.

Examination of the chemical-shift perturbations of SpoIIAAS58D allows the effect of a charge at residue 58 on the structure of SpoIIAA to be determined. Since this mutant was assigned in its monomeric form, there are no additional perturbations due to dimerization. Figure 2(A) shows the significant chemical-shift perturbations of SpoIIAAS58D from SpoIIAA. There were fewer chemical-shift perturbations in this mutant than in SpoIIAA-phosphate, whose spectrum includes perturbations caused by dimerization. Apart from a single residue in helix 2, all the significant perturbations in SpoIIAAS58D were in helix 3 and its surrounding loops.

The dimerization site of SpoIIAAS58D can be identified by determining which resonances, observed in the monomer, were significantly reduced in intensity. Figure 2(B) shows, in blue, the position of residues with such resonances. Figure 2(B) also shows, in red, residues which have significant chemical-shift perturbations. Residues with both significantly reduced intensity and significant chemical-shift perturbations are shown in purple. Comparison of Figure 2(B) with Figure 1 shows that the combination of dimerization (as seen by reduction in intensity) and structural perturbation due to the introduction of a charge in SpoIIAAS58D (as seen by chemical-shift perturbation), largely accounts for the chemical-shift perturbations in SpoIIAA-phosphate.

The chemical-shift perturbations in SpoIIAAS58D may be compared with the differences found between the crystallographic structures of SpoIIAA and SpoIIAA-phosphate from B.
Structural changes on SpoIIAA phosphorylation

Figure 6 Titration of the histidine side chains of SpoIIAA, SpoIIAA-phosphate and SpoIIAAS58D

C$_2$H chemical shifts are shown as a function of sample pH, and were fitted to a modified Hill equation.

*sphaericus* [4]. Figure 3 shows the crystallographic structure of SpoIIAA superimposed on that of SpoIIAA-phosphate; the phosphorylation-induced changes in structure are subtle and occur mainly in helix 3 and its surrounding loops. These structural changes correlate strongly with the chemical-shift differences between SpoIIAAS58D and wild-type, suggesting that the addition of a charge at the phosphorylation site significantly perturbs similar regions in solution and crystal state.

We have shown previously that the charge-neutral mutant SpoIIAAL90A (with a Leu$^90$ → Ala mutation) has subtle structural differences from wild-type SpoIIAA, which result in a > 100-fold increase in its dissociation constant from SpoIIAB [7,12]. The significant chemical-shift perturbations of SpoIIAAL90A are shown in Figure 4. A comparison of Figure 2(A) and Figure 4 shows that the chemical-shift perturbations in SpoIIAAS58D are of a similar magnitude and occur in a similar region of the molecule to those in SpoIIAAL90A. Therefore the structural perturbations induced by a change in charge at the phosphorylation site appear to be similar to those that occur in SpoIIAAL90A and alter its SpoIIAB-binding properties.

**Histidine titration studies**

In the vicinity of the phosphorylation site, there are two adjacent histidine residues (residues 24 and 25) that are strongly conserved in SpoIIA-like proteins [4,13]. The positions of these two histidine residues in the structure are shown in Figure 5, together with the positions of other conserved charged residues in the SpoIIAA family that are near the phosphorylation site. To investigate the effects of phosphorylation, we measured the histidine pK$_a$ values by means of 1H NMR pH titration studies. The C$_2$H chemical shifts of the three histidines were determined across the pH range 5–8.5 in SpoIIAA, SpoIIAA-phosphate and SpoIIAAS58D. The results are shown in Figure 6, with curves fitted with a modified Hill equation (see the Experimental section), and the values generated by the curve-fitting procedure are given in Table 1. Residue 45, which is an internal histidine, gives, as expected, a low pK$_a$ value and an h value of approx. 1. The pK$_a$ and h values remain unchanged in the three different forms of the protein. In contrast, histidines 24 and 25 have higher pK$_a$ values and, most strikingly, unusually low h values. Such low h values are a result of the titrating species interacting with other titrating groups, which causes their protonation to occur across a particularly extended range of pH values. As shown in Figure 5, the side chains of these histidines are closely aligned, suggesting that interactions between them could contribute to their low h values. A further group in close proximity is the aspartate residue at position 23. The pK$_a$ value of residue 24 is higher in SpoIIAA-phosphate than in SpoIIAA by 0.4 of a pH unit, and SpoIIAAS58D has an intermediate value. Such an increase in the pK$_a$ value indicates that the histidine residue is interacting with
Chemical shift

$pK_a$

It is further from the negative charge. The $pK_a$ of residue 25 is increased only slightly in the SpoIIAA-phosphate group with charged residues, such as histidine, may be sufficient to induce the observed structural perturbations in helix 3 and its surrounding loops.

**DISCUSSION**

**Effects of phosphorylation on SpoIIAA**

We have shown that, in solution, subtle structural changes, mainly in helix 3 and its surrounding loops, occur when a charge is introduced at the phosphorylation site. We have used results obtained with SpoIIAAS58D to unravel phosphorylation and dimerization effects. Although the structure of SpoIIAAS58D is not identical with that of SpoIIAA-phosphate (two clearly differ in their ability to dimerize), it is likely to have similar structural changes from the non-phosphorylated wild-type as observed in SpoIIAAS58D, has a dissociation constant from those observed in SpoIIAAS58D; it has a dissociation constant from SpoIIAB at least 100-fold higher than that of the wild-type [7,12]. These findings demonstrate that SpoIIAA is required to be in a very specific conformation at its binding site to form a complex with SpoIIAB, and that minor structural changes can substantially alter its binding affinity, even in the absence of a charge effect. We therefore suggest that the subtle structural perturbations seen in SpoIIAA on phosphorylation act in concert with the change in charge to alter the binding affinities of this protein.

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<th>Table 1</th>
<th>NMR titration studies of histidines</th>
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<tr>
<td>Property</td>
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<tr>
<td>$pK_a$</td>
<td>24       25     45</td>
</tr>
<tr>
<td>SpoIIAA</td>
<td>5.82 ± 0.01</td>
</tr>
<tr>
<td>SpoIIAA-phosphate</td>
<td>6.23 ± 0.04</td>
</tr>
<tr>
<td>SpoIIAAS58D</td>
<td>6.66 ± 0.15</td>
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$h$

|  SpoIIAA       | 0.71 ± 0.02 | 0.65 ± 0.01 | 1.02 ± 0.25 |
| SpoIIA-phosphate | 0.66 ± 0.04 | 0.57 ± 0.02 | 1.16 ± 0.16 |
| SpoIIAAS58D   | 0.66 ± 0.12 | 0.57 ± 0.02 | 1.11 ± 0.10 |

Chemical shift

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<th>Protonated</th>
<th>Unprotonated</th>
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<tr>
<td>$+$</td>
<td>8.60</td>
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<tr>
<td>$-$</td>
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</tr>
<tr>
<td>$+$</td>
<td>8.24</td>
<td>7.84</td>
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a negative group. We can conclude that residue 24 interacts with the phosphate in SpoIIAA-phosphate and the aspartate in SpoIIAAS58D, since these are the only new negative charges that have been introduced into this region. The $pK_a$ value of residue 25 is increased only slightly in the SpoIIAA-phosphate and mutant SpoIIAAS58D forms, presumably because it is further from the negative charge. The $h$ values of both residue 24 and residue 25 are lower in SpoIIAA-phosphate and SpoIIAAS58D than in SpoIIAA, which is also consistent with the formation of new interactions with the phosphate or aspartate groups.
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