The NMR determination of the IIA^{mtl} binding site on HPr of the *Escherichia coli* phosphoenol pyruvate-dependent phosphotransferase system

Nico A.J. van Nuland, Gerard J.A. Kroon, Klaas Dijkstra, Gea K. Wolters, Ruud M. Scheek and George T. Robillard

The BIOSON Research Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

The region of the surface of the histidine-containing protein (HPr) which interacts with the A domain of the mannitol-specific Enzyme II (IIA^{mtl}) has been mapped by titrating the A-domain into a solution of ^{15}N-labeled HPr and monitoring the effects on the amide proton and nitrogen chemical shifts via heteronuclear single quantum correlation spectroscopy (HSQC). Fourteen of the eighty-five HPr amino acid residues show large changes in either the ^{15}N or ^{1}H chemical shifts or both as a result of the presence of IIA^{mtl} while a further seventeen residues experience lesser shifts. Most of the residues involved are surface residues accounting for approximately 25% of the surface of HPr. Phosphorylation of HPr with catalytic amounts of Enzyme I (EI), in the absence of IIA^{mtl} resulted in chemical shift changes in a sub-set of the above residues; these were located more in the vicinity of the active site phospho-histidine. Phosphorylation of the HPr/IIA^{mtl} complex resulted in a HSQC spectrum which was indistinguishable from the P-HPr spectrum in the absence of IIA^{mtl} indicating that, as expected, the complex P-HPr/P-IIA^{mtl} does not exist even at the high concentrations necessary for NMR.

Nuclear magnetic resonance; Transport system; Enzyme II; P-HPr; P-IIA^{mtl}; HSQC-spectroscopy

1. INTRODUCTION

The P-enolpyruvate-dependent phosphotransferase system in prokaryotes couples the transport of hexoses and hexitols to their phosphorylation at the expense of P-enolpyruvate. To this end, a sequence of five phosphoryl group transfer steps occurs: P-enolpyruvate → P-EI → P-HPr → P-IIA → P-IIB → sugar-P. IIA and IIB are cytoplasmic components of the membrane-bound transport protein [1]. For the mannitol-specific enzyme II in *E. coli*, the IIA and IIB domains are covalently linked to the membrane-bound C domain, IIC. We have separated these domains at the gene level to understand the reason for this coupling and to determine the structure of the various domains. Part of the structural work involves determining the solution structure of Pr [2], IIA^{mtl} and IIB^{mtl} and their phosphorylated species and the complexes between them using multidimensional NMR. NMR is the method of choice for

Abbreviations: NMR, nuclear magnetic resonance; HMQC, heteronuclear multiple quantum correlation; HSCQ, heteronuclear single quantum correlation; HPr, histidine-containing protein; EI, Enzyme I; EII, Enzyme II; Mtl, mannitol; IIA, IIB, IIC, the A, B and C domains of Enzyme II; DTT, dithiothreitol; TPPI, time-proportional phase incrementation; rf, radio frequency; NOE, nuclear Overhauser enhancement

studying the structure of the phosphorylated intermediates because these intermediates hydrolyze at physiological pH and must be continually regenerated with the help of EI, a process which cannot be realized in a crystal. The method provides a similar advantage in determining the structure of protein complexes since it circumvents the difficulties associated with crystallization of these complexes.

2. MATERIALS AND METHODS

2.1. Protein purification

The production and purification of IIA^{mtl} was carried out as described elsewhere [3]. The protein was dialyzed against 5 mM potassium phosphate buffer, pH 7.3 and freeze-dried in portions of 4 and 7 mg. Uniformly ^{15}N-enriched HPr was produced and purified as previously described [4]. EI was purified following published procedures [5]. It was dialyzed against 25 mM potassium phosphate pH 7.3, 2 mM DTT and lyophilized. Protein concentrations were determined using the pyruvate burst method [5].

HSQC spectra were recorded at pH 7.3 in 50 mM potassium phosphate buffer. Phosphorylation of HPr in the absence of IIA^{mtl} was accomplished in situ using 50 mM P-enolpyruvate, 2.5 μM EI, 5 mM MgCl$_2$ and 3 mM DTT in a 50 mM potassium phosphate buffer at pH 7.3. Before and after each experiment the pH was checked (pH 7.3 ± 0.1).

2.2. NMR measurements

The HSQC experiments [6,7] were performed on a Varian Unity 500 MHz NMR spectrometer. All experiments were run at 20°C. ^{1}H chemical shifts are expressed relative to DSS, ^{15}N chemical shifts are expressed relative to liquid NH$_3$ [8]. Time-proportional phase incrementation (TPPI) [9] was used to discriminate between positive and

negative $\omega 1$ (^{15}N) frequencies. Typical experimental details: maximum $t1$ and $t2$ values were 128 ms and 154 ms, respectively, and spectral widths in the $\omega 1$ (^{15}N) and $\omega 2$ (^{1}H) domains were 2000 Hz and 6666 Hz. The ^{1}H carrier was at the water resonance, the ^{15}N carrier in the middle of the spectrum. During the acquisition period, ^{15}N-decoupling was achieved by a broadband Waltz decoupling sequence using a radio frequency (rf) field of $\gamma B1 = 1$ kHz. The water resonance was suppressed by a short presaturation pulse (20 ms), and by inserting a 5 ms trim pulse before the sond 90° proton pulse. The delay. τ, was tuned to 2.3 ms, about 20% shorter than $\frac{1}{4}J_{NH}$.

^{31}P-NMR spectra were recorded on a Varian VXR 300 at 20°C using a repetition rate of 5 s. The intensity ratios of the phosphorylated protein signals was constant at repetition rates of 5 s and higher.

3. RESULTS

Fig. 1A presents a HSQC spectrum of uniformly ^{15}N-labeled *E. coli* HPr. It contains all of the peaks identified in our earlier heteronuclear multiple quantum correla-

tion (HMQC) spectrum which dealt with the sequential assignments [4]. We have chosen the amide of Gly-13 indicated at the top of the spectrum to illustrate the changes observed during a gradual titration of IIA^{mtl} into the HPr solution. Fig. 1B presents the Gly-13 amide cross peak from the HSQC spectra at the indicated IIA^{mtl}/HPr molar ratios. The peak shifts with each addition of IIA^{mtl} up to a molar ratio of 1.4. In the middle of this titration it also decreases in intensity suggesting possible exchange broadening. Fig. 1C presents the lowest contour of each of the peaks in 1B at their correct ^{1}H and ^{15}N chemical shifts. A plot of the absolute chemical shifts as a function of the IIA^{mtl}/HPr molar ratio in Fig. 1D shows that the titration is essentially complete at a molar ratio of 1. Before addition of the last portion of IIA^{mtl}, the IIA^{mtl}/HPr was diluted with an equal volume of buffer to determine whether
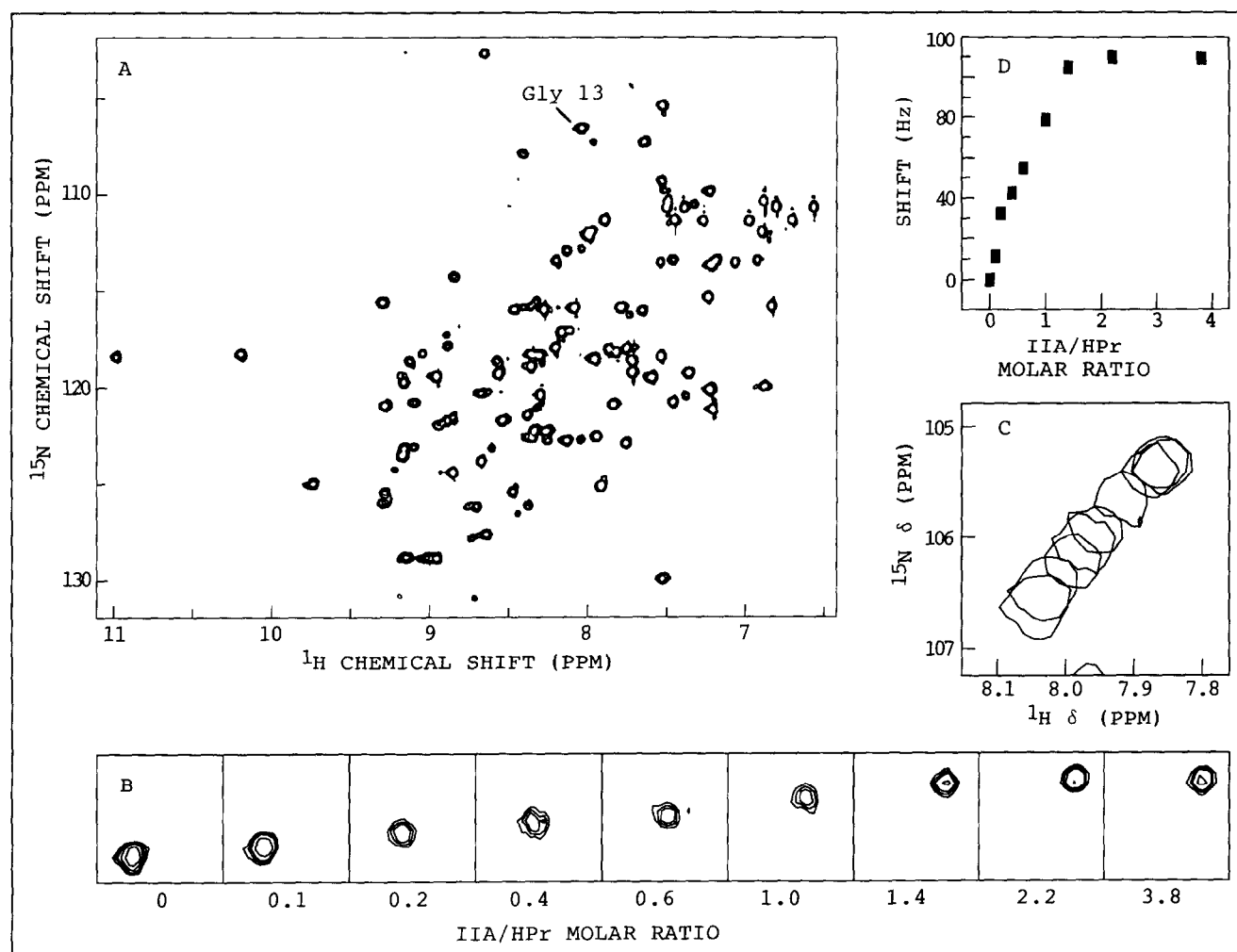

Fig. 1. Changes in the HSQC spectrum illustrated for Gly-13 to show the effects during the gradual titration of IIA^{mtl} to ^{15}N-labeled HPr. A. The HSQC spectrum of HPr at 20°C and pH 7.3. The HPr concentration was 1.25 mM. The ^{1}H-^{15}N cross peak corresponding to Gly-13 is indicated. B. The Gly-13 cross peak from the HSQC spectra at the indicated IIA/HPr molar ratios. Note the decreasing intensity in the middle of the titration suggesting possibly exchange broadening. C. Summary of the lowest contours given in Fig. B at their correct ^{1}H and ^{15}N chemical shifts (δ). D. The absolute shifts in Hz as a function of the IIA/HPr ratio. The shift is defined as the distance over which the ^{15}N-^{1}H cross peak is displaced on adding IIA.

dilution would cause the complex to dissociate and the peaks to shift back to their earlier positions. No changes in the spectrum occurred after dilution.

Plots of the changes in the ^{15}N and ^{1}H chemical shifts of each amide in the IIA^{mtl}/HPr complex at the end of the titration are shown in Fig. 2. Positive and negative shifts up to 1 ppm for ^{1}H and up to 4 ppm for ^{15}N occur. The solid block corresponding to residues 15, 16 and 17 indicates that the cross peaks of these amides disappeared in the complex. The top section of Fig. 2 plots the ^{15}N/^{1}H chemical shift vector for each amide cross peak. It is clear that the residues 13 to 21 and 48 to 56 are the most strongly effected by the complexation with IIA^{mtl}. At the pH of these measurements, the cross peak corresponding to the backbone Asn-38 ^{1}H-^{15}N spin pair is not observed. Therefore, this position has been left open in Fig. 2.

Complexation also leads to measurable changes in chemical shifts of side chain amide cross peaks. Even though the backbone amide of Asn-12 is hardly effected by complexation, the lowfield-shifted side chain ^{1}H-^{15}N spin pair shifts 0.23 ppm (^{1}H) and −0.11 ppm (^{15}N), corresponding to an absolute shift of 115 Hz. A similar change (78 Hz) is observed for the lowfield-shifted side chain amide cross peak of Asn-38. Both side chain NH_2 cross peaks of Gln-51 disappeared on complexation. The highfield-shifted side chain ^{1}H-^{15}N spin pairs of Asn-12 and Asn-38 as well as all other side chain NH_2 groups of the glutamines 3, 4, 21, 57 and 71 were hardly effected (shifts < 35 Hz) by complexation.

Since P-HPr rather than HPr is the substrate for

IIA^{mtl} (with a K_m of P-HPr for EII^{mtl} of 4–15 μM [10]) we examined the effects of phosphorylation on the HSQC spectrum of HPr. The number of amides effected is fewer than in the case of the IIA^{mtl}/HPr complex. Substantial changes in chemical shifs occur only for residues close to the phosphorylation site, indicating that the effects of phosphorylation are more localized to the active site. Only the lowfield-shifted side chain ^{1}H-^{15}N spin pair of Asn-12 is substantially effected, 0.13 ppm (^{1}H), 0.23 ppm (^{15}N), corresponding to an absolute shift of 64 Hz. All other side chain NH_2 groups are hardly effected by phosphorylation. No changes occurred on addition of P-enolpyruvate alone (data not shown). During the subsequent in situ hydrolysis of the phospho-histidine of P-HPr the shifted cross peaks gradually disappeared and reappeared at their known chemical shift positions in unphosphorylated HPr. Due to this slow-exchange behaviour we could not be certain about some of the amide ^{1}H and ^{15}N assignments in P-HPr. Nevertheless, the P-HPr HSQC spectrum could be used to study the effects of phosphorylation of both HPr and IIA^{mtl} on the stability of the complex. P-HPr was prepared in situ and the HSQC spectrum was recorded. Subsequently, P-IIA, prepared separately, was added to the sample to a molar ratio of 1.6. No chemical shift changes in the initial HSQC spectra were observed beyond those observed in the P-HPr spectrum alone. Hydrolysis of the phospho-proteins and rephosphorylation however, gradually depleted the P-enolpyruvate supply. Once the supply was reduced to the point where the rate of in situ phosphorylation could not keep up
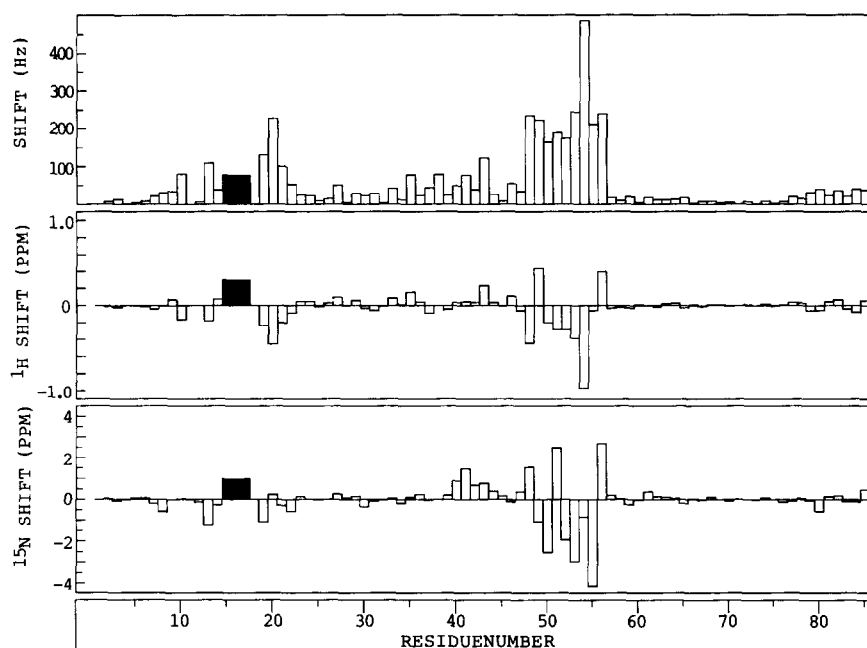

Fig. 2. Plots of the change in ^{15}N and ^{1}H chemical shift observed at the end of the titration with IIA versus the residue number of the amide ^{1}H-^{15}N spin pair. Black bars at residues 15, 16 and 17 indicate that the corresponding ^{1}H-^{15}N cross peak disappeared after addition of IIA. Bottom, ^{15}N shifts; middle, ^{1}H shifts; top, the absolute shift as defined in the legend to Fig. 1D.
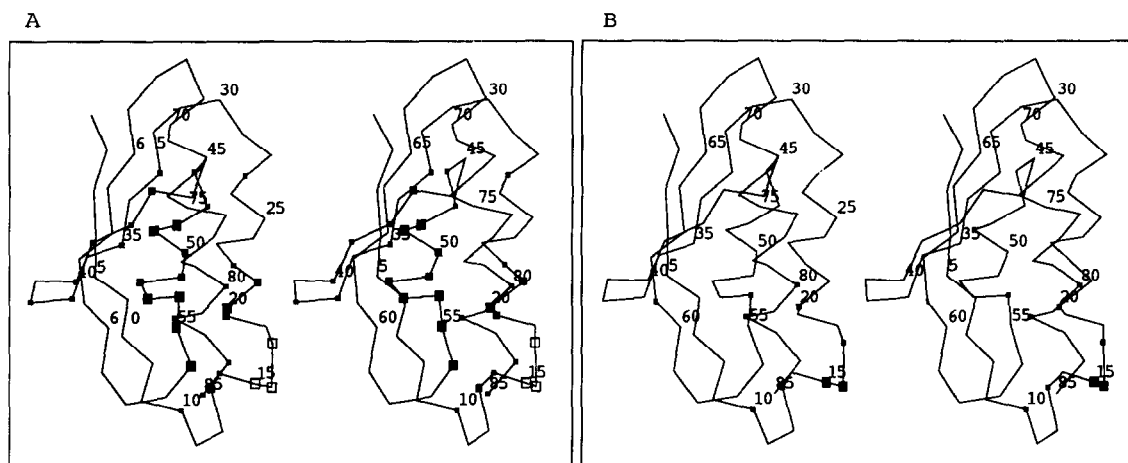
Fig. 3. Summary of the chemical shift changes observed at the end of the titration with IIA (A) and on phosphorylation of HPr (B) in a stereoscopic representation showing the α-carbon positions in the molecule. Every fifth amino acid is labeled. Residues with shifts between 35 and 100 Hz are indicated by small black squares, those with shifts between 100 and 200 Hz by medium sized black squares, and those with shifts bigger than 200 Hz are indicated by large black squares. Blank squares in Fig. A indicate residues for which the ^{1}H-^{15}N cross peaks disappeared after adding IIA to HPr. The black square at the Cα position 38 in Fig. A corresponds to the shift of the lowfield-shifted side chain ^{1}H-^{15}N spin pair of Asn-38.

with dephosphorylation, the HSQC spectrum started to change and eventually reverted back to a spectrum identical to that observed for the non-phosphorylated HPr/IIA^{mtl} complex. The state of phosphorylation during this experiment was intermittently monitored by ^{31}P-NMR. From this experiment we conclude that the HPr/IIA^{mtl} complex is destabilised when both His-15 in HPr and His-65 in IIA^{mtl} are phosphorylated. The phosphohistidine on P-HPr must position itself in the active site of IIA^{mtl} in such a way that the phosphoryl group can be transferred to the active site histidine on IIA^{mtl}. If both active sites histidines are phosphorylated, these phosphoryl groups will repel each other and prevent a P-HPr/P-IIA^{mtl} complex from forming. The above results also indicate that the shifts observed in the spectrum of the HPr/IIA^{mtl} complex are due to specific interactions of the binding site. If the shifts were due to non-specific interactions between regions other than the binding site, repulsion from the phosphoryl groups would be less likely and the shifts resulting from the non-specific interactions would be observed in the phosphorylated complex as well.

4. DISCUSSION

The 3D structure of HPr has been the center of considerable attention in the past two years. The solution structure of *Bacillus subtilis* HPr has been studied by NMR [11] and the crystallographic structure has been solved to 2 Å resolution [12]. In the case of *E. coli* HPr, crystallographic techniques have not yet yielded a reliable structure, however, the NMR spectrum has been assigned [4,13] and a low resolution structure generated from NMR NOE data using distance geometry and restrained molecular dynamics algorithms [2]. The to-

pology is consistent with the recently published structure of *B. subtilis* HPr [12].

A stereoscopic illustration of *E. coli* HPr is presented in Fig. 3A. The effects of complexation on the amide resonances can be divided into four categories: i, loss of the resonances; ii, vector shifts larger than 200 Hz; iii, vector shifts between 100 and 200 Hz; iv, vector shifts between 35 and 100 Hz. The amides of His-15, Thr-16 and Arg-17, represented by open squares, disappear upon addition of IIA^{mtl}. Large black squares indicate amides which experience vector shifts larger than 200 Hz. Those with shifts between 100 and 200 Hz are indicated by medium sized black squares and those with shifts between 35 and 100 Hz by small black squares. The most prominent effects of complexation are found on the adjacent 5 residues on both sides of the active site His-15 and in the loop and helix containing residues 35 to 56. The loop is one of the most poorly defined regions in the NMR structure indicating possible conformational flexibility which may be essential for adaptation of this portion of HPr to the binding sites of its various partners such as EI and A domains of other sugar-specific enzymes II.

Close inspection of the residues which experience shifts as a result of complex formation show that they are not all facing the surface of HPr. In a number of cases they are found on the inner side of helical segments which form contacts with the underlying β-sheet, while other residues in the same segments face outward and presumably form contacts with IIA^{mtl}. Apparently binding induces structural rearrangements between different elements of the HPr tertiary structure.

Phosphorylation has remarkably little effect on the chemical shifts when compared with IIA^{mtl} binding. Although uncertainties remain about some amide assign-

ments in P-HPr, it is certain that the major effect of phosphorylation is on residues close to His-15. One region which does experience shifts of roughly the same magnitude in both data sets are residues 79 to 85. These residues lie on the C-terminal α-helix which begins after the turn containing Glu-68 at the top of the structure in Fig. 3. Van Dijk et al. [14] showed that replacement of this glutamic acid by alanine shifted the equilibrium for the phosphoryl group self-exchange reaction between HPr and the E68A mutant in favor of native HPr. The chemical shift changes of residues in this helix upon phosphorylation and complex formation and the mutagenesis results suggest a role for this helix in the phosphorylation/ dephosphorylation sequence [15,16].

The present data deals only with the effects of phosphorylation and complex formation on chemical shifts. A wealth of information is available in the effects of these events on amide proton exchange rates, ^{15}N and ^{1}H relaxation rates and ^{15}N-^{1}H hetero-NOEs. The fact that the amides of residues 15, 16 and 17 as well as those of the Gln-51 side chain disappear upon complex formation suggests increased amide-water exchange rates or multiple conformations with intermediate rates of exchange or altered relaxation properties. Experiments are in progress to extract and interpret this additional information and to complete the assignments in the P-HPr spectrum.

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