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Structural and functional NMR studies on HPr from Escherichia coli

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Summary

The phosphoenolpyruvate-dependent phosphotransferase system (PTS) in prokaryotes couples the transport of hexoses and hexitols to their phosphorylation at the expense of phosphoenolpyruvate. To this end, a sequence of five phosphoryl group transfer steps occurs between *P*-enolpyruvate *P*-EI *P*-HPr *P*-IIA *P*-IIB sugar-*P*. Enzyme I (EI) and the histidine-containing phosphocarrier protein HPr are both cytoplasmic proteins. IIA and IIB are cytoplasmic components of the membrane-bound transport protein enzyme II. For the mannitol-specific enzyme II in *Escherichia coli*, the IIA and IIB domains are covalently linked to the membrane-bound C domain, IIC. These domains have been separated 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 HPr, IIA^{mtl} and IIB^{mtl} and their phosphorylated species and the complexes between them using multi-dimensional Nuclear Magnetic Resonance (NMR). NMR is the method of choice for studying the structure of the phosphorylated intermediates because these intermediates hydrolyse at physiological pH and must be continually regenerated with the help of EI, a process which cannot be realised in a crystal. The method provides a similar advantage in determining the structure of protein complexes since it circumvents the difficulties associated with crystallisation of these complexes. The protein of interest in this thesis is HPr. It plays a unique role in the phosphorylation cascade because it has to accept and transfer phosphoryl groups from and to different enzymes, involved in different sugar-specific transport processes, and therefore it must be able to adapt its binding interface to various partners.

Structural details can be obtained from Nuclear Overhauser Effect (NOE) spectra. In these spectra cross-peaks are observed between protons which are within a certain distance (mostly smaller than 0.5 nm) in three-dimensional (3D) space, and therefore provide direct structural information about the protein of interest. To assign these cross-peaks to a certain pair of protons, one has to label each proton in the protein with a frequency at which it resonates in a magnetic field. The first step in this assignment procedure involves the sequential assignment. Chapter 2 reports the complete assignments for the backbone ¹H, ¹⁵N and ¹³C resonances, using 3D heteronuclear ¹H NOE ¹H-¹⁵N multiple-quantum coherence spectroscopy and 3D heteronuclear total correlation ¹H-¹⁵N multiple-quantum coherence spectroscopy experiments on ¹⁵N-enriched HPr and an additional 3D triple-resonance ¹HN-¹⁵N-¹³Ca correlation spectroscopy experiment on ¹³C, ¹⁵N-enriched HPr. Many of the sequential backbone ¹H assignments, as

derived from two-dimensional (2D) NMR studies, previously reported by Klevit and co-workers, were corrected.

Chapter 3 describes the side-chain assignments and the first structure calculations on HPr. Several types of heteronuclear 3D NMR spectra on ^{15}N -enriched and $^{13}\text{C},^{15}\text{N}$ -enriched HPr were recorded to extend the backbone assignments to the side-chain ^1H , ^{15}N and ^{13}C resonances. From both 3D heteronuclear ^1H NOE $^1\text{H}-^{13}\text{C}$ and ^1H NOE $^1\text{H}-^{15}\text{N}$ multiple-quantum coherence and 2D homonuclear NOE spectra, more than 1200 NOEs were identified and used in a step-wise structure refinement process using distance geometry (DG) and restrained molecular dynamics (MD) involving a number of new features. A cluster of nine low-resolution structures, each satisfying the set of NOE restraints, resulted from this procedure. The secondary structure topology of the molecule is that of a classical open-face β -sandwich formed by four antiparallel β strands packed against three α helices. In addition to the conventional NOE-patterns and J-couplings used to identify secondary structure elements, the chemical shifts of the $^1\text{H}\alpha$, $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ nuclei appear to be useful probes for secondary structure analysis.

Chapter 4 and part of Chapter 7, shows that by performing relatively simple NMR experiments on isotope enriched protein, it is possible to determine the binding interface for various partners of the protein of interest in a very straightforward, fast way, without knowing the structural details of the various partners. Chapter 4 describes the region of the surface of HPr which interacts with the A domain of the mannitol-specific enzyme II. The region has been mapped by titrating the A-domain into a solution of ^{15}N -labelled HPr and monitoring the effects on the amide proton and nitrogen chemical shifts via heteronuclear single quantum correlation spectroscopy. Fourteen of the eighty-five HPr residues show large changes in either the ^{15}N or ^1H chemical shifts or both as a result of the presence of IIA^{mtl} while a further seventeen residues experience lesser shifts. Phosphorylation of HPr with catalytic amounts of 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. The binding site for EI on HPr resembles that of the IIA^{mtl} , as was concluded in Chapter 7. Changes on the amide proton and nitrogen chemical shifts were observed for the residues which were also sensitive to the presence of IIA^{mtl} .

Chapters 5, 6 and 7 report the structural changes which occur at the active-site of HPr upon phosphorylation. Structures of both the unphosphorylated and phosphorylated forms were obtained by the use of NMR in combination with DG

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and restrained MD in water. Distances derived from NOE spectra recorded on HPr and P-HPr were used in a time-dependent manner during the MD simulations in water to attain experimental agreement. The HPr structure was found to be very similar to the X-ray structures of *Bacillus subtilis* and *E. coli* HPr. A unfavourable ϕ torsion angle was found for residue 16 in the active centre of unphosphorylated *Streptococcus faecalis* HPr. These authors proposed that the strain is released upon phosphorylation, as evidenced by the lack of strain in the *B. subtilis* X-ray structure, where a sulphate ion was found to be co-crystallised in the active centre, and therefore was suggested to represent the phosphorylated form of HPr. Although present at early stages of the structure calculations of unphosphorylated HPr, this torsion-angle strain disappeared in the final model obtained from MD simulations in water using time-averaged distance restraining and upon releasing the distance restraints. This suggests that the strain may have been an artefact of the crystallisation conditions instead of an essential element in the phosphorylation/dephosphorylation process. Structural changes upon phosphorylation of HPr are limited to the active site, as evidenced by changes in chemical shifts, in $^3J_{\text{NH}\alpha}$ -coupling constants and NOE patterns. Chemical shift changes were obtained mainly for protons which were positioned close to the phosphoryl-group attached to the His15 imidazole ring. Differences could be detected in the intensity of the NOEs involving the side chain protons of His15 and Pro18, resulting from a change in the relative position of the two rings. In addition, a small change could be detected in the three-bond J -coupling between the amide proton and the $\text{H}\alpha$ proton of Thr16 and Arg17 upon phosphorylation, in agreement with the changes of the ϕ torsion angle of these two residues obtained from time-averaged restrained MD simulations in water. The proposed role of the torsion-angle strain at residue 16 in the mechanism of *S. faecalis* HPr is not supported by these results. In contrast, phosphorylation introduces torsion angle strain at the His15, suggesting a mechanism in which part of the phosphoryl-group chemical energy is stored as conformational energy in P-HPr.

Chapter 5 showed that there was hardly any overlap between the clusters of structures obtained at the different stages of the refinement protocol, telling us that each cluster samples a different region of the conformational space. This reflects the fact that members of the different clusters were subjected to different force-fields: the simple geometric force field of the DG protocol, the *in vacuo* GROMOS force field including distance restraining of the MD protocol, and the GROMOS force field including explicit waters, with and without time-averaged distance restraining. The most complete force field, including solvent and combined with time-averaged distance restraining, is expected to lead automatically to the most accurate model of the HPr molecule. Thus far, we have performed two such simulations and had to conclude from an analysis of the two

resulting clusters that, also during these two simulations, different regions of the conformational space were sampled. We therefore concluded that it remains necessary to perform a number of such simulations, starting from different starting conformations, in order to obtain a representative sampling of the conformations adopted by the molecule under the experimental conditions, and that distance restraining remains necessary to obtain complete agreement with the experimental data. Chapter 8 describes the solution behaviour of HPr in more detail. Three unrestrained MD trajectories starting from three different starting conformations, were analysed using a recently developed technique to separate the protein's total motion into two subspaces: a low-dimensional essential subspace, in which most of the protein's motion occurs in a highly correlated, very anharmonic fashion, and the remaining subspace, where the motion can be described as rapidly equilibrating, harmonic near constraints. The restrained MD trajectories were projected onto these essential and near-constraints subspaces to see how the simulated motion of HPr was effected by NOE-derived distance restraints. The mean square displacements along the essential co-ordinates decreased significantly due to the distance restraints, even when the distance restraints were applied in a time-dependent manner. The restraints which were violated during the free MD simulations were partially responsible for the inhibition of the essential large scale motions. Only after the restraints were released, was the protein able to enter regions of the conformational space that were inaccessible given the restraints and the way they were imposed. Resume: (i) distance restraining limits the accessible conformational space because motions that occur on time scales larger than the averaging period will be missed; (ii) without distance restraining the sampling problem remains, because presently available computers only allow realistic MD simulations of proteins over time spans of nanoseconds. The low dimensionality of the essential subspace can now be used to design an efficient algorithm for sampling a protein's conformational space.