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Aspartyl phosphates in the regulatory control of bacterial response

Review Article

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Summary. Acyl phosphates represent a mixed anhydride class of compounds whose lability allows the phosphorylation of an aspartyl carboxyl contained in a protein to reversibly induce changes in structure that may have biological significance, particularly in prokaryotic systems. In this report the phosphorelay system that regulates sporulation in *Bacillus subtilis* is described briefly and its analogy to other regulatory systems is outlined. The structural properties of the aspartate containing second component of the phosphorelay system SpoOF is examined by multidimensional NMR techniques and comparison is made with a known sequence analog, CheY. Distinct differences are apparent that are reflected by the extended half life of phosphorylated SpoOF relative to the CheY analog. It is probable that in a general way the distinct half life characteristics may be related to the differing functions of the various regulatory aspartate containing proteins in the cell.

Keywords: Amino acids – Aspartyl phosphates – Phosphorylation, *Bacillus subtilis* – Multi-dimensional NMR – Regulation

Introduction

Acyl phosphates, because of their comparative instability, have until recently been somewhat neglected targets of investigation in biochemistry. However, during the past five years it has become apparent that the phosphorylation of an aspartyl group contained in an amino acid sequence constitutes a unique switching mechanism for regulatory proteins in bacteria (Bourret et al., 1989; Stock et al., 1990; Hoch, 1993; Strauch, 1993). The action appears to have similarities to the formation of phosphate esters of tyrosine, serine and threonine in eukaryotic cellular regulation (Edelman et al., 1987; Hunter and Cooper,

1985), but the inherent properties of the acyl phosphate mixed anhydride bond ensure that the prokaryotic or bacterial regulatory action is distinctly different. Usually the formation of such a phosphorylated derivative is governed by a signal, either external or internal, that incites a kinase to autophosphorylate on a histidine residue using ATP as the source of phosphate. This kinase then transfers its phosphate to the aspartate containing regulatory protein which may undergo a conformational change that induces interaction with a genomic promoter region or promotes the transfer of the phosphate further down a regulatory sequence of protein intermediates. Bacteria contain several dozen of these switches which allow them to respond to a spectrum of stimuli that relate to nutrition, metabolic deficiencies, the acquisition of virulence or even the development of resistance to antibiotics. As a model for bacterial regulatory action this laboratory is currently exploring the structural and mechanistic features of the sporulation controlling phosphorelay in *Bacillus subtilis* that determines whether or not the bacterium undergoes vegetative growth or enters stationary phase and sporulation. The phosphorelay is made up of four proteins known as KinA, SpoOF, SpoOB and SpoOA (Fig. 1). KinA is a histidine kinase that autophosphorylates using ATP and passes the phosphate to SpoOF, an aspartate containing protein, that then conveys the phosphate to SpoOB, a further histidine containing protein, and finally the phosphate is passed to SpoOA, which is a further aspartate containing protein containing two domains. The N-terminal domain is closely related to SpoOF in sequence and probably structure, however, the second domain contains the regulating component that acts on the abrB gene and stimulates the overall bacterial response. This report summarises pertinent properties of acyl phosphates and describes our approach to the structural characterisation of SpoOF. Some attempt will be made to indicate how these structural features may relate more generally to other bacterial regulatory systems.

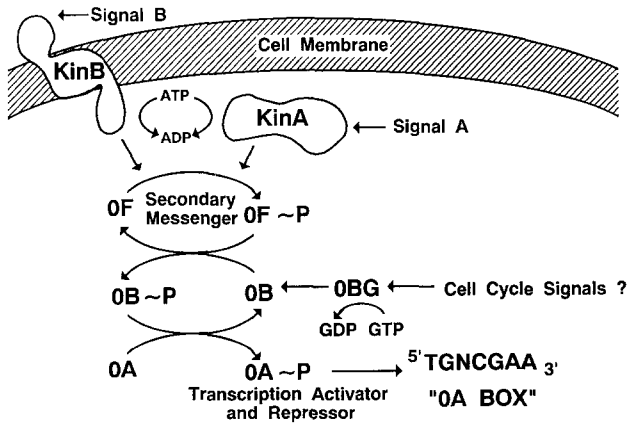

Fig. 1. The phosphorelay system of *B. subtilis* showing the interrelationship of KinA, SpoOF, SpoOB and SpoOA. A second kinase KinB is also indicated, however, this has not yet been fully characterised. In addition the possible regulatory signals from a protein OBG are shown and the promoter sequence of the abrB gene is indicated ("OA Box")

Properties of acyl phosphates

Acyl phosphates are usually prepared *in vitro* by elimination reactions which lead to the exclusion of H_2O or RX from the respective carboxylic acid and phosphoric acid derivatives, where X is a halide and R a suitable cation, and the products are precipitated as salts with minimal aqueous contact (Black and Wright, 1955; Yamaguchi et al., 1980). These procedures are designed to

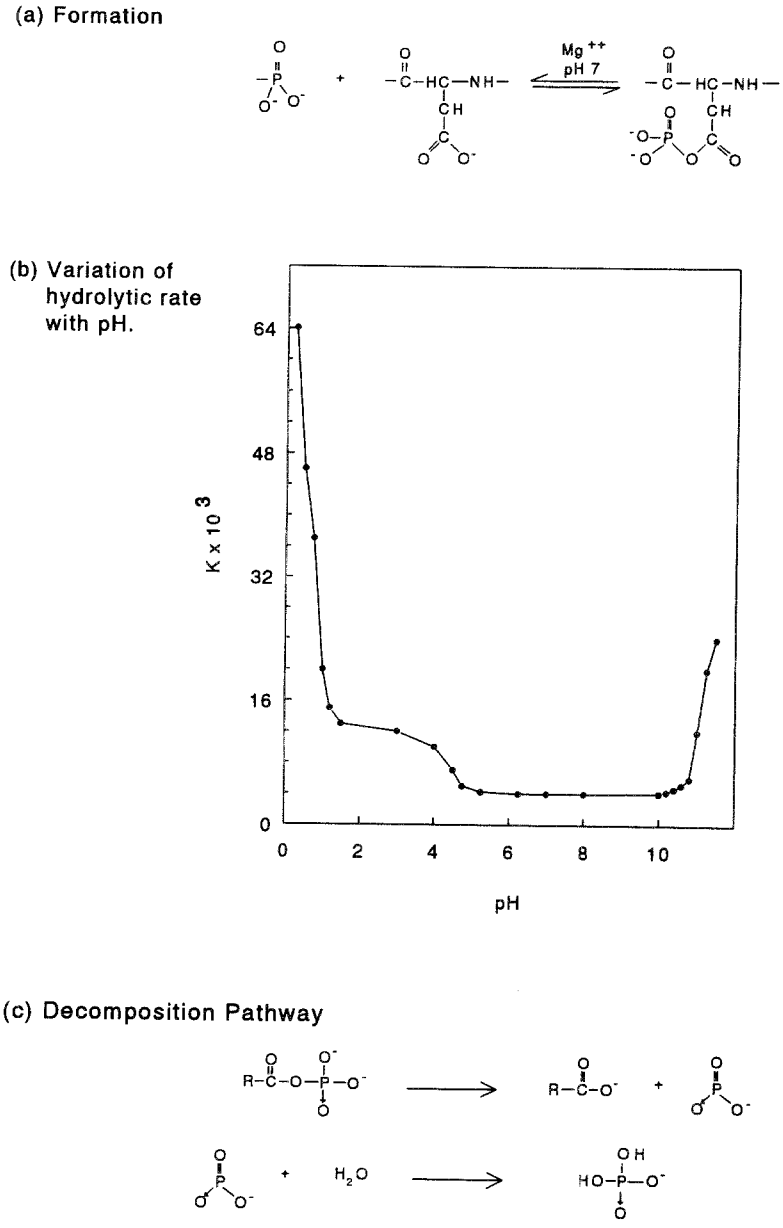

Fig. 2. Characteristics of acyl phosphates: **a** Preparative mode illustrating the transfer of phosphate from the histidine kinase, KinA, to the aspartate containing SpoOF at pH 7. **b** The rate of decomposition of acetyl phosphate. **c** The prevalent dissociation pathway of acyl phosphates at pH 7 showing P—O bond scission

minimise hydrolysis, which can occur rapidly in aqueous solution. When the acyl function is aspartate, suitable protecting groups for the α-carboxyl and amino groups are necessary (Black and Wright, 1955). However, when the aspartyl group is contained in a regulatory protein such as SpoOF then phosphorylation is induced by interaction with KinA and ATP and in addition magnesium ions are required (Fig. 1) (Burbulys et al., 1991). The biological phosphorylation is usually carried out at pH ~ 7 and despite the necessity for an aqueous medium the hydrolysis of the product can vary considerably which presumably is dependent on the environment of the aspartyl phosphate group in the phosphorylated protein. Thus phosphorylated SpoOF has a $t_{1/2}$ of several hours at room temperature and pH 7, whereas a closely analogous protein, CheY when phosphorylated, has $t_{1/2}$ the order of minutes.

The pH rate of hydrolysis curve included in Fig. 2 is taken from Koshland (1952), and shows the rate of decomposition of acetyl phosphate over the full pH range. It is clear that both base and acid accelerate decomposition with a rate decrease occurring around pH 4.5 which corresponds to the generation of the dianion. A similar curve is also observed with aspartyl phosphate (Black and Wright, 1955) although the rates of hydrolysis of this compound are somewhat higher. The decomposition of the acyl phosphate can occur by the breakage of C—O or P—O bonds (Di Sabato and Jencks, 1961a). *In vitro* with the simple acyl phosphates a break in the P—O linkage tends to occur, particularly around neutral pH. Cleavage is accentuated in the presence of tertiary amines and pyridine, however, it would appear that the rules for cleavage are somewhat flexible in the presence of nitrogen bases (Di Sabato and Jencks, 1961b). Dications also accelerate cleavage of the acyl phosphates. In the sporulation regulatory pathway the mode of favored hydrolysis appears to be P—O bond scission as transfer of phosphate can be to the histidine of SpoOB or a return to the histidine of KinA. The known phosphatase activity associated with the regulatory process could invoke either C—O or P—O bond scission but at present which pathway is taken is unknown.

The sporulation controlling phosphorelay

The general cellular features of the phosphorelay in *B. subtilis* are shown schematically in Fig. 1. The genes of each of the components, KinA, SpoOF, SpoOB and SpoOA, have been cloned and associated proteins overexpressed in *Escherichia coli* (Trach et al., 1988). This system offers several features that make it attractive for investigation. Each of the components is soluble and the kinase does not contain transmembrane components in its N-terminal sequence. In addition the activity of the sequential phosphorylation can be readily monitored on SDS polyacrylamide gels using radioactively labeled ATP (Burbulys et al., 1991). The first two components of the relay also show considerable sequence homology to equivalent proteins in a large number of bacterial two component regulatory response systems (Stock et al., 1990), and moreover of particular importance is the observation that SpoOF has an M_r ~ 14,200, which allows its structural investigation by both X-ray crystallography and high resolution multidimensional NMR. The relative stability of the phosphorylated SpoOF

molecule is also important as it will make possible the structural identification of the aspartyl phosphate containing protein.

As stated earlier SpoOF is a close analog of CheY both in sequence and molecular weight. The latter's crystal structure has been reported by two laboratories (Stock et al., 1989; Volz and Matsumura, 1991) and an interesting feature of the structure is that three aspartyl groups residing at residues no. 12, 13 and 57 are united by virtue of the protein folding to form an accessible pocket on the surface of the protein and it is at this site that phosphorylation occurs. Mass spectral studies have suggested association is ultimately with Asp57 (Sanders et al., 1989), however, it is possible that this derivative is the final stable species of the structural investigation, but is not the transient active species essential for *in vivo* viability. The short half life of CheY-P gives rise to significant practical difficulties when its structure is examined. In contrast the relative stability of SpoOF-P and its relatively small molecular weight make it an attractive model for all aspartyl phosphate receptors of this type and therefore multidimensional NMR studies are being pursued to characterise first SpoOF, then SpoOF-P.

Characterization of SpoOF

An efficient two step purification procedure has been developed for isolating SpoOF from the *E. coli* expression system and the product, homogeneous by HPLC and SDS polyacrylamide gel electrophoresis, is regularly obtained in 100 mg quantities and can be concentrated to the 2 mM solutions necessary for NMR. In addition using minimal media growth conditions uniformly labelled ^{15}N and doubly labelled ^{13}C, ^{15}N samples have been obtained.

Using a Bruker instrument excellent quality spectra at 500 MHz for protons are regularly obtained as shown in Figs. 3–5. Initially, purely ^{1}H NMR spectra of SpoOF were recorded, but these proved to be of little value due to excessive chemical shift overlap problems. In order to improve resolution and so remove ambiguities in the spectra, the two isotopically labeled samples of SpoOF were used, the one incorporating ^{15}N, the other both ^{15}N and ^{13}C. There are two main advantages of using such isotopically labeled species. Firstly, both ^{15}N and ^{13}C offer much improved chemical shift dispersion compared to ^{1}H only spectra and so it is possible to alleviate most, if not all, resonance congestion problems. Secondly, many multi-dimensional NMR experiments which utilize the larger through bond scalar couplings present in such heteronuclear systems (J_{NH}, J_{CH} and J_{CC}) are often much more efficient and sensitive than homonuclear (^{1}H only) methods.

Figure 3 contains the results of a 3D-HCCH-TOCSY experiment designed to identify specific amino acid side chains within the sequence. In this figure the characteristic patterns which can be attributed to residue types Asp and Ile have been identified. The ^{15}N 3D-TOCSY-HSQC spectrum is used for both amino acid side chain identification and for resonance assignments for amide protons and amide nitrogens and can be correlated with the 3D-HCCH-TOCSY spectrum through the alpha and beta proton resonances. Following the identification of the individual amino acid types, these systems need to be linked sequentially.

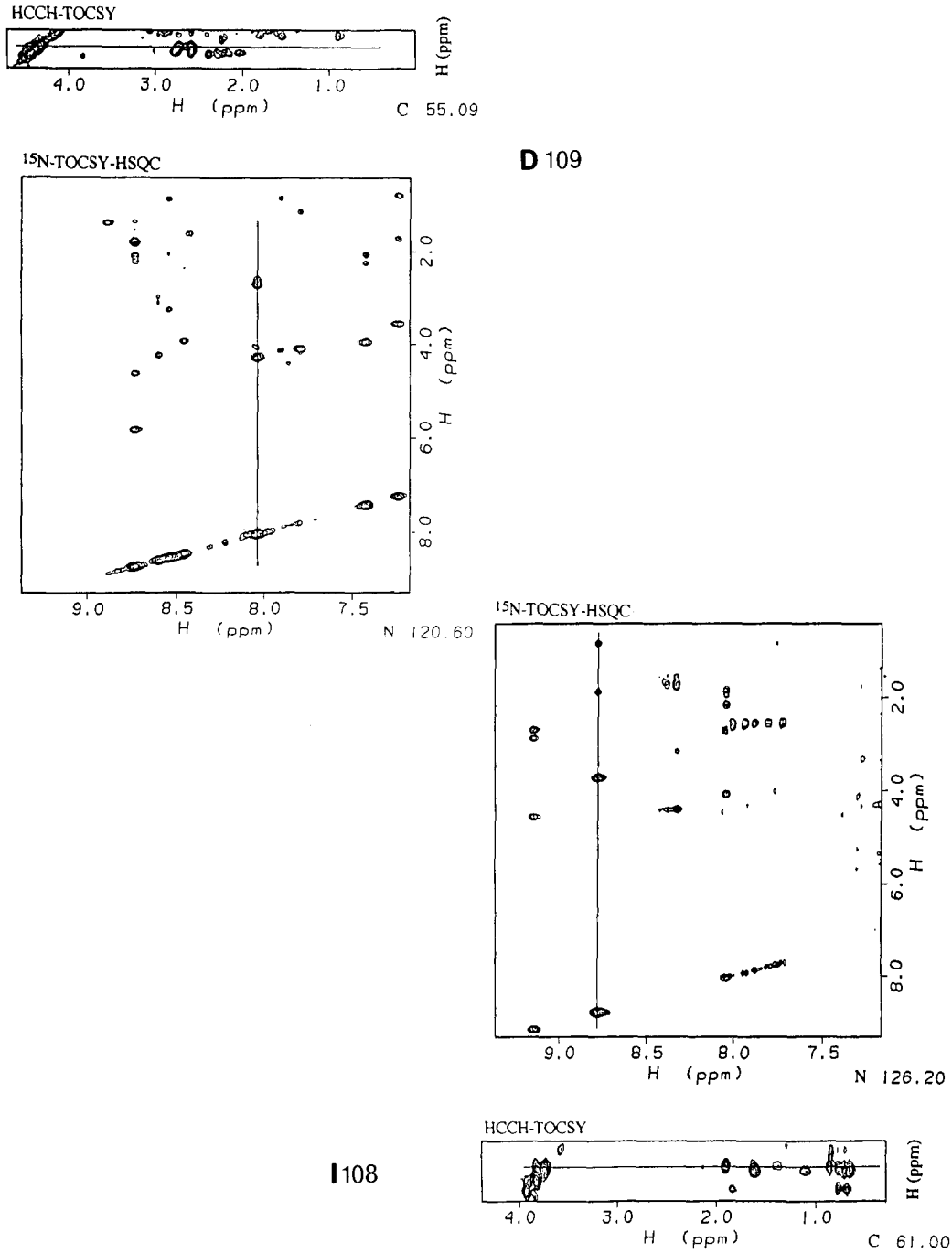

Fig. 3. HCCH-TOCSY and ^{15}N-TOCSY-HSQC NMR spectra indicating the characteristic contour patterns assigned to amino acids Ile 108 and Asp 109. The sample used was 1.5 mM, 300°K, pH = 6.8 in a 90:10 H_2O:D_2O mixture

To do this, two 3D triple resonance NMR experiments were employed. The 3D HNCA experiment is used to link the amide proton, amide nitrogen and alpha carbon of a single amino residue. The 3D HN(CO)CA experiment is used to link the amide proton and amide nitrogen of one amino acid residue with the alpha

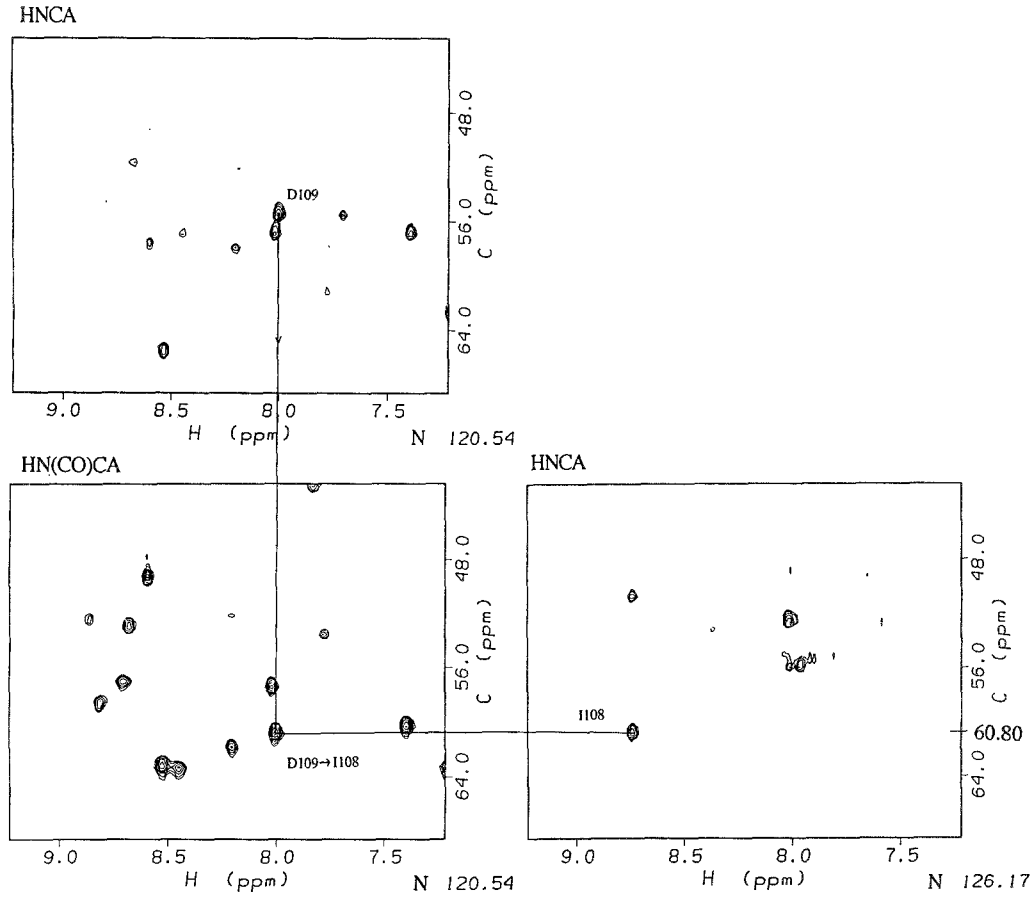

Fig. 4. HNCA and HN(CO)CA spectra illustrating the sequential interaction of Ile 108 and Asp 109. Sample was at 1.2 mM, 300°K, pH = 6.8, in 90:10 H_2O:D_2O

carbon of the *preceding* amino acid residue. The combination of these two experiments allows the polypeptide backbone to be sequentially assigned. Figure 4 shows the linking of residues Asp 109 and Ile 108 by this method. Using this procedure repeatedly allows the sequence to be recognised. It is then important to determine secondary structural information by using through space interactions via the nuclear Overhauser enhancement in the form of NOESY experiments and an example of the application of this technique is shown in the strip plots of Fig. 5 for helix A. The helix and many loop regions for SpoOF have been identified and Fig. 6 shows a comparison of the CheY and SpoOF structures.

It is apparent from these results that subtle structural differences exist between SpoOF and CheY. For example helix A is shifted in SpoOF relative to the adjacent aspartates at positions 10 and 11. This suggests a more extended loop region may exist connecting β sheet 1 to helix A if comparison is made with the known CheY crystal structure (Stock et al., 1990). This may allow the aspartates greater conformational freedom at the active site. In addition, pursuing the comparison, β sheet 3 must be closely attached to helix B to ensure Asp 54 is correctly oriented to participate as the third component of the active

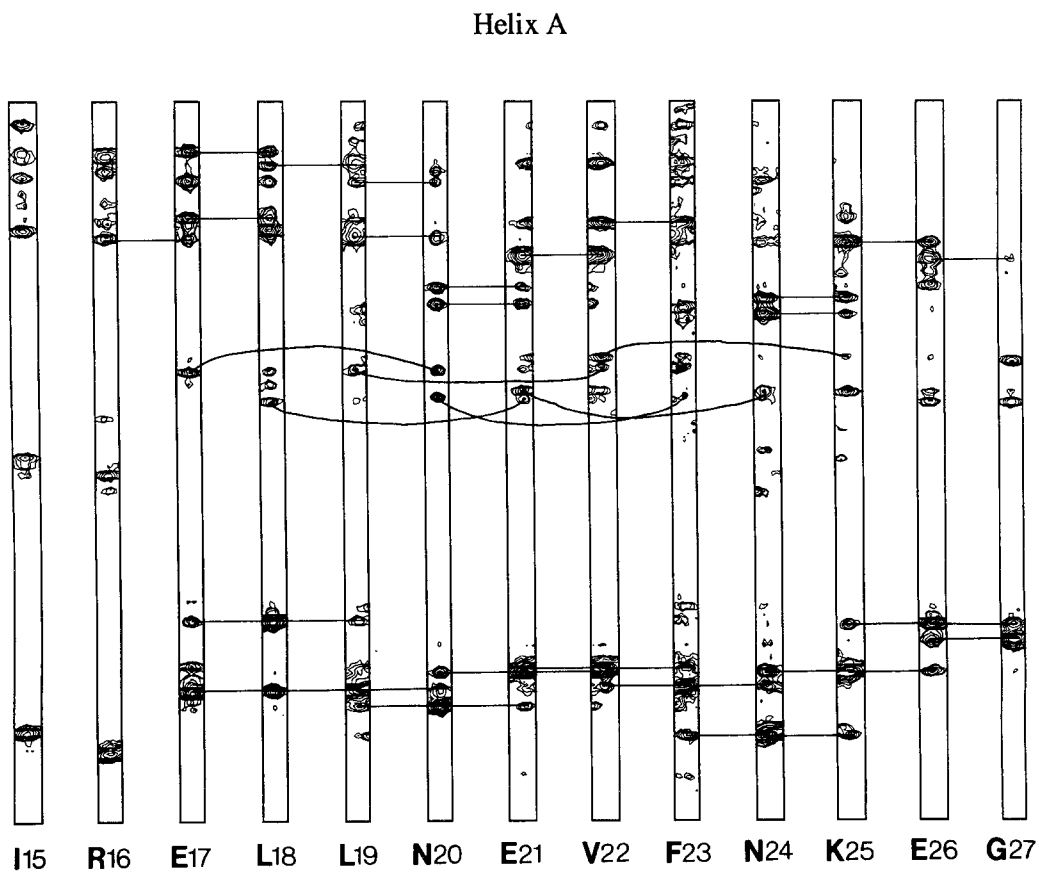

Fig. 5. A strip-plot of NOE connectivities from a nitrogen 3D-NOESY-HSQC spectrum. The presence of d_{NN} (i, i + 1) and $d_{\alpha N}$ (i, i + 3) NOE connectivities (bottom and middle connected contours respectively) are indicative of an alpha helix. Also shown are the $d_{\beta N}$ (i, i + 1) interactions (top). The pattern correlates with residues Ile 15 to Asn 24 identified as Helix A. Experimental conditions were outlined in Fig. 4

site. This again assumes that helix B in both CheY and SpoOF have comparable lengths. The final participant in the active region, Lys 104, in the SpoOF numbering sequence, at present would appear to have equal freedom in both molecules. The regional changes for helices D and E may reflect the differing requirements of the two molecules in the phosphorylation switching process but at present more definitive statements cannot be made.

As inferred above, multidimensional NMR was required because the globular symmetrical nature of the folded protein with hydrophobic core and hydrophilic exterior led to considerable resonance overlap. However, the dynamic nature of the NMR experiment has clear advantages when protein-protein or protein-substrate interactions are considered. In fact titrations with Mg^{++} and examination of the phosphorylated protein have shown significant resonance shifts in preliminary experiments. This identification of the exact structural identity of the protein aspartyl phosphate binding site is currently underway as the understanding of its exact function might explain the features that enhance or decrease the stability of the mixed anhydride function *in vivo*

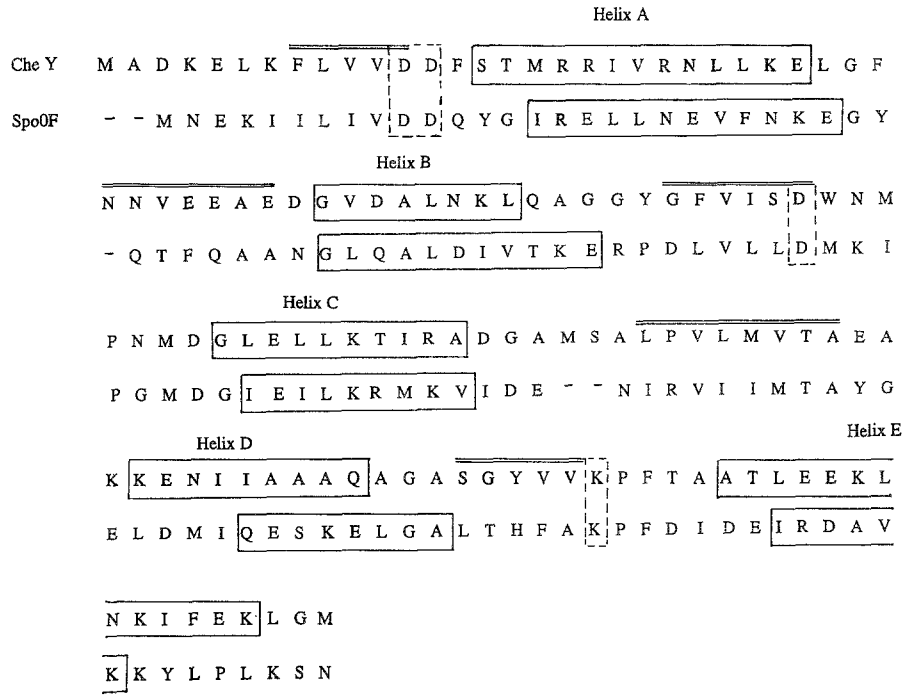

Fig. 6. Comparison of the helix and loop regions determined for SpoOF by multidimensional NMR with those reported for CheY. The β-strand regions of CheY are illustrated by a double line and amino acids associated with the active site are enclosed by dashed boxes

and then explain the differential biological action exhibited by the many regulatory systems now recognised.

Corollary

The unique properties of the aspartyl phosphate linkage allow its use as a biological regulatory switch. The energy content of the acyl phosphate linkage is similar to that of the phosphoramidate and therefore facile transfer can occur between protein containing histidine phosphates and aspartates. The wide occurrence of the switching process for a great number of bacterial regulatory actions testifies to the importance of the reaction sequence. Of note is the observation that virulence of bacterial pathogens is controlled by such a sequence and so is even the development of the cell. The sequence similarity of kinases and regulatory proteins and their apparent common mechanism of action coupled with their sequences having little in common with eukaryotic proteins suggests that they may be targets for drug action in a manner distinct from current known families of antibiotics. Moreover, it has been reported that the development of resistance to the antibiotic vancomycin results from the action of a two component switch (Arthur et al., 1992). Thus it is clear that a fundamental understanding of the aspartyl phosphate transfer mechanism in the sporulation system will aid in a general understanding of regulatory proteins. Moreover, in the sporulation system itself the analogy between the N-terminal sequences of SpoOA and SpoOF will perhaps allow an understanding at the

molecular level of how the second domain of the former protein can be influenced by N-terminal phosphorylation of the first domain and thus how gene regulation may occur.

Acknowledgements

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References

Arthur M, Molinas C, Courvalin P (1992) The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J Bacteriol 176: 2582–2591

Black S, Wright NG (1955) β-Aspartokinase and β-aspartyl phosphate. J Biol Chem 213: 27–38

Bourret RB, Hess JF, Borkovich KA, Pakula AA, Simon MI (1989) Protein phosphorylation in chemotaxis and two-component regulatory systems of bacteria. J Biol Chem 264: 7085–7088

Burbulys D, Trach KA, Hoch JA (1991) Initiation of sporulation in *B. Subtilis* is controlled by a multicomponent phosphorelay. Cell 64: 545–552

Di Sabato G, Jencks WP (1961a) Mechanism and catalysis of reactions of acyl phosphates. II. Hydrolysis. J Am Chem Soc 83: 4400–4405

Di Sabato G, Jencks WP (1961b) Mechanism and catalyses of reactions of acyl phosphates. I. Nucleophilic reactions. J Am Chem Soc 83: 4393–4400

Edelman AM, Blumenthal DK, Krebs EG (1987) Protein serine/threonine kinases. Ann Rev Biochem 56: 567–613

Hoch JA (1993) Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. Ann Rev Microbiol 47: 441–465

Hunter T, Cooper JA (1985) Protein tyrosine kinases. Ann Rev Biochem 54: 897–930

Koshland DE (1952) Effect of catalysts on the hydrolysis of acetyl phosphate. Nucleophilic displacement mechanisms in enzymatic reactions. J Biol Chem 74: 2286–2292

Sanders DA, Gillece-Castro BL, Stock AM, Burlingame AL, Koshland DE (1989) Identification of the site of phosphorylation of the chemotaxis response regulator protein, CheY. J Biol Chem 264: 21770–21778

Stock AM, Mottonen JM, Stock JB, Schutt CE (1989) Three-dimensional structure of CheY, the response regulator of bacterial chemotaxis. Nature 337: 745–749

Stock JB, Stock AM, Mottonen JM (1990) Signal transduction in bacteria. Nature 344: 395–400

Strauch MA (1993) Regulation of *Bacillus subtilis* gene expression during the transition from exponential growth to stationary phase. Prog Nucl Acid Res Mol Biol 46: 121–153

Trach K, Chapman JW, Piggot P, Lecoq D, Hoch JA (1988) Complete sequence and transcriptional analysis of the *spoOF* region of the *Bacillus subtilis* chromosome. J Bacteriol 170: 4194–4208

Volz K, Matsumura P (1991) Crystal structure of *Escherichia coli* CheY refined at 1.7 Å resolution. J Biol Chem 266: 15511–15519

Yamaguchi K, Kamimura T, Hata T (1980) A method for the preparation of unesterified acyl phosphates via stannyl phosphate intermediates. J Am Chem Soc 102: 4534–4536

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