

STRUCTURE AND MAGNESIUM BINDING OF PEPTIDE FRAGMENTS OF LHCII IN ITS PHOSPHORYLATED AND UNPHOSPHORYLATED FORMS STUDIED BY MULTINUCLEAR NMR.

Dalibor Stys¹, Torbjörn Drakenberg², Michael D. Spangfort³, Sture Forsén² and John F. Allen¹.

¹Plant Cell Biology, Box 7007, Lund University, S-220 07 Lund, Sweden,

²Physical Chemistry 2, and ³Biochemistry, Lund University, Box 124, S-221 00 Lund, Sweden

1. Introduction

It is believed that upon phosphorylation, some LHCII complexes move towards unappressed regions of the thylakoid and act as light-harvesting antennae for photosystem I [1,2,3]. A structure at 3.4 Å resolution has been described for the major part of non-phosphorylated LHCII [4] but gives no information regarding the N-terminal domain that contains the phosphorylation site. Attempts to isolate pure phosphorylated LHCII have so far been unsuccessful.

Appression of thylakoid membranes, also called "stacking", is induced by the presence of cations. For a long time there has been a dispute about the involvement of specific and non-specific interactions in this process [1,2,3,5,6]. Barber [1] suggested the dominance of unspecific electrostatic interactions in both stacking and lateral segregation of proteins, Mullet and Arntzen [8] considered dominance of protein-protein interactions involving LHCII in stacking and Allen [3] proposed dominance of protein-protein interactions in both stacking and lateral segregation. If unspecific, electrostatic shielding of electrostatic repulsion between lamellae in the presence of salts induces appression, then the salt concentration may be several orders of magnitude lower in the interlamellar space than in the rest of the solution [7]. This prediction is in clear disagreement with experimental evidence from thylakoids, where the membranes, although sometimes highly appressed, are more enriched in cations than it would be needed for compensation of the surface charge. The electrostatic equilibrium concentration may be restored by EDTA treatment, by which tight complexes may be disrupted. An alternative hypothesis could thus be proposed by which the interlamellar interactions in stacking are dominated by specific binding of ions rather than by purely electrostatic effects.

It is known that LHCII has a strong tendency to aggregate [5]. The three-dimensional structure of the stromal part of LHCII [4,8] is relatively flat and contains a number of carboxylic acid groups. It may also contain a phosphatidylglycerol (PG) headpiece which is known to be tightly bound to LHCII [9]. Several molecules of digalactosyldiacylglycerol (DGDG) are necessary for proper crystallisation of LHCII [8]. Carboxylic acids, phosphate groups and saccharides are known to bind cations.

A number of laboratories have produced data on the relative effectiveness of mono- and di-valent cations in cation-dependent processes in thylakoid membranes and in lipid vesicles. There is an apparent similarity of the effectiveness of Mg^{2+} relative to monovalent cations for stacking of thylakoids [10], for aggregation of LHCII [5], and for association of PG vesicles [11]. Such similarity may be just coincidental and the whole phenomena needs proper analysis.

In systems containing a number of non-equivalent binding sites, the occupancy of binding site is determined by the local activity of ions [11]. The local activity is determined by electrostatics, by the affinity of neighbouring specific binding sites and by the position of individual binding sites on the surface [11]. However, in equilibrium conditions, the activity of cations should be the same all over the whole sample. In the case of thylakoids, a strong ion binding site is introduced when a phosphate group is attached to a membrane protein. We may well assume non-equilibrium conditions in thylakoid membranes, as it is known from fractionation experiments that it takes at least 30 min for the membrane to equilibrate for new conditions. Thus, a timescale for the equilibration exceeds that of phosphorylation-induced charges and local non-equilibrium conditions may be expected.

In the few known examples of magnesium binding to phosphate groups on protein [12,13] the binding constants are always of the order of few hundreds to thousands M^{-1} , largely exceeding the binding constant of the phosphate group of lipids [11,14]. Here we describe results obtained with small synthetic peptide fragments of the LHCII phosphorylation site. In any case the incorporation of the phosphate group into a protein at the membrane surface will significantly affect the binding of ions at weaker sites.

2. Results

2.1 Comparison of NMR spectra of the phosphorylated and non-phosphorylated LHCII fragments

Major differences between the spectra of the non-phosphorylated and phosphorylated peptides are clearly observed at all pH values (Figure 1). We will therefore concentrate the discussion to the spectra at pH 5.3, where the assignment of resonances was least complicated by spectral overlap and by exchange of NH protons.

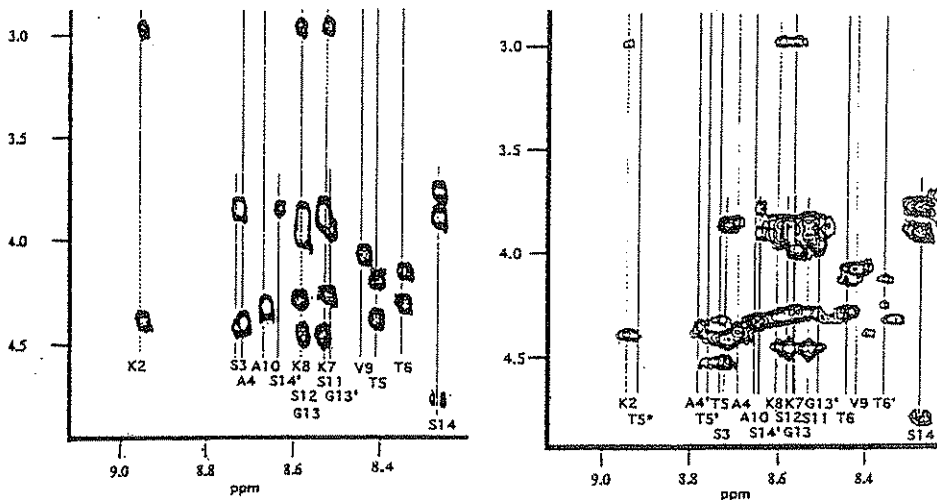


Figure 1. Segments of 1H NMR TOCSY spectrum of unphosphorylated LHCII fragment and of phosphorylated LHCII fragment at pH 5.3

Both the non-phosphorylated and phosphorylated forms of the peptide RKSAT(PO₃)TKKVASSGSP show non-random-coil chemical shifts for most of the protons (Figure 1). Major differences in chemical shifts between the two forms of the peptide are observed for protons of Thr 5 and 6, and these can be attributed to changes in

covalent structure and bond properties. In addition, changes in chemical shifts are greater in the region Ser 3 - Lys 8 than in the C-terminal part of the molecule. This indicates that changes in structure (or stabilisation of one prevalent structure) occur in the N-terminal part of the molecule upon phosphorylation. Further strong evidence for the existence of a different preferred structure in each form of the peptide is the observed doubling of the resonances of Ser 14 and Gly 13 in the non-phosphorylated form and in those of Ala 4, Thr(PO₃) 5 and Thr 6 in addition to Ser 14 and Gly 13 in the phosphorylated form. This doubling is most likely caused by *cis/trans* isomerism around the peptide bond between Ser 14 and Pro 15. The doubling of resonances for residues 4 to 6 shows that there exists at least one more populated conformation in which the C-terminus is sufficiently close to residues 4 to 6 to affect their chemical shifts. In addition, several non-sequential mainchain-to-sidechain NOE crosspeaks are seen in the spectrum of the phosphorylated peptide whereas in the non-phosphorylated peptide NOESY crosspeaks are observed only between the sidechains of Lys 7 or Lys 8 and Ser 14 NH. There is, however, a clear indication of a preferred tertiary structure in the non-phosphorylated peptide between residues 7 and 15, and of extension of such a structure right up to the N-terminus of the phosphorylated peptide.

The pH-dependent changes in the spectrum were consistent with pK_a's of 4.5 and 5.9 for the single and double protonations, respectively, of the phosphate group, consistent with previous data on the protonation of the phosphate group of various phosphoproteins given in [15].

2.2 Magnesium binding to peptides

The binding constant of Mg²⁺ ions to both phosphorylated and unphosphorylated peptide was determined from the concentration dependence of line broadening of the ²⁵Mg NMR signal at 25°C (Fig.2).

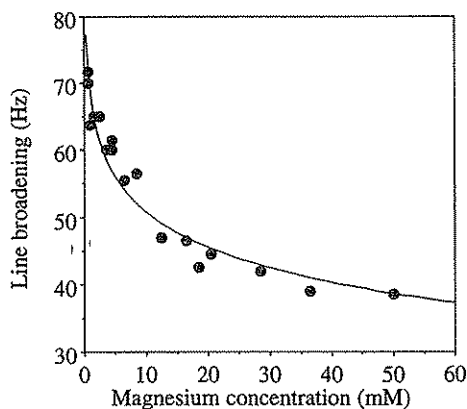


Figure 2. Dependency of line broadening of ²⁵Mg²⁺ signal in presence of 2.1 mM phosphorylated LHCI fragment on concentration of Mg²⁺.

No magnesium binding site is located on the unphosphorylated peptide. In contrast the phosphorylated peptide binds magnesium. The calculation of the binding constant of Mg²⁺-phosphopeptide complex gives a value of 110 M⁻¹. The binding constant is low in comparison to known binding constants of phosphate group which are between 250 and 25000 M⁻¹ in casein and casein-derived peptides [12] where the magnesium binding is to cluster of serine phosphates. The binding constants to phosphate groups on lipids and sugars are, however, of the order of 5-20 M⁻¹.

Proton NMR spectroscopy was performed in 8 mM solution of the phosphorylated peptide in concentrations of $MgCl_2$ that were increased stepwise up to 150 mM. Small differences were found for the chemical shifts of the NH protons. The differences in chemical shift were uniform in direction, varying between 0.04 and 0.01. In the work of Wahlgren et al. [13] changes in chemical shifts were found also for the α protons surrounding the metal-binding phosphoserines. These changes [13] were one order of magnitude larger than those found in our work, indicating that the magnesium-induced change in the structure is very small, if any.

3. Conclusions

The proton NMR spectra of the phosphorylated as well as the unphosphorylated peptide gave indications of the existence of a preferred structure in solution. Marked differences between the proton NMR spectra of the phosphorylated and unphosphorylated fragments were observed. These differences may be interpreted either as a change in preferred structure or as a stabilisation of one of the preferred conformations of the N-terminal part of the peptide. The structure of the peptide furthermore varied with changes in the protonation of the phosphate group.

The phosphate group on proteins is known to be one of the strongest magnesium binding groups in biochemical systems [14]. The hypothesis [16] that stacking of thylakoid membranes is determined to a large extent by binding of magnesium to lipids seems to have some experimental support [5,9,11]. The binding constant of $110 M^{-1}$ for magnesium to the phosphopeptide is almost one order of magnitude higher than the value $12 M^{-1}$ found in the literature for PG [11]. In phosphorylated LHCII will the phosphate group at the N-terminus favourably compete with the sites on lipids unless the phosphate group is buried in the structure.

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