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International Journal of Mass Spectrometry 219 (2002) 681–696

International Journal of Mass Spectrometry

www.elsevier.com/locate/ijms

Electrospray ionization mass spectrometry analysis revealed a ∼310 kDa noncovalent hexamer of HPr kinase/phosphatase from *Bacillus subtilis*

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Received 4 March 2002; accepted 29 April 2002

Dedicated to Dr. Yannik Hoppilliard for her participation in the development of mass spectrometry during her carrier.

Abstract

The benefits of electrospray ionization mass spectrometry (ESI-MS) to measure large noncovalent complexes were used to determine the oligomeric state of the bifunctional enzyme HPr kinase/phosphatase (HPrK/P) from *Bacillus subtilis*. This enzyme is involved in the main regulatory mechanism of carbon catabolite repression, i.e., the hierarchical control of carbohydrate utilization. It was unambiguously shown that HPrK/P is a specific noncovalent homohexamer of ∼310 kDa at pH 6.8. A detailed study of the experimental conditions required to perform high molecular weight mass measurements is presented. Especially dissociation of the noncovalent edifice was induced by subjecting the ions to increasing gas phase collisions either by varying the pressure in the interface region of the mass spectrometer (P_i) or the accelerating voltage (V_c), which were shown to be crucial parameters for the detection of high *m*/*z* ions. The fact that pH variations induced strong changes on the ESI mass spectra provided a high level of confidence for a "structurally-specific" hexamer. Supramolecular mass spectrometry presents several benefits towards classical methods for the characterization of very large biological noncovalent assemblies: among those are its high accuracy, sensitivity and rapidity. (Int J Mass Spectrom 219 (2002) 681–696)

Keywords: Noncovalent complex; Electrospray ionization mass spectrometry; HPr kinase/phosphatase; Protein/protein interactions

1. Introduction

Most enzymes exist in their active form as oligomers, most commonly as dimers or tetramers [1,2]. A major advantage attributed to oligomerization is the improved stability of the multimeric enzymes, for example to thermal denaturation or to the presence of denaturing agents [3–6]. Another advantage may lie in the possibility of allosteric interactions between the subunits: in some multimeric enzymes, these interactions give rise to cooperative binding of substrates, which is believed to play a key role in the regulation of their enzymatic activity [7,8]. Common techniques used to determine the oligomerization state of a protein include gel exclusion chromatography

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1387-3806/02/$ – see front matter
PII S1387-3806(02)00747-9

or analytical ultracentrifugation. However, a precise quantification and an accurate determination of the molecular weight of oligomeric proteins are not directly and precisely available with those techniques.

In this context, we evaluated the possibilities offered by electrospray ionization mass spectrometry (ESI-MS) to measure the mass of large noncovalent complexes and therefore to probe the oligomerization state of HPr kinase/phosphatase (Histidine-containing protein kinase/phosphatase—HPrK/P) from *Bacillus subtilis*. This enzyme is involved in the carbon catabolite repression (CCR) mechanism found in several low-GC (guanine, cytosine) Gram-positive bacteria. CCR induces the alteration of specific catabolic genes and thus allows the bacteria to metabolize different carbon sources in a hierarchical way. Metabolizing a preferred carbohydrate, such as glucose, prevents the expression of catabolic genes required for the utilization of secondary carbon sources and affect the synthesis of enzymes, including both inhibition and activation of genes, implicated in central metabolic pathways, such as glycolysis, as well [9–12]. To date one expects a high oligomerization state for HPrK/P to play a key role in the regulation of its enzymatic activity. Supramolecular mass spectrometry, which deals with the investigation of edifices maintained by noncovalent interactions, seems to be particularly attractive and well-suited to go further inside into the characterization of the oligomeric form of this enzyme.

In the last few years, ESI-MS has been increasingly used to study supramolecular edifices formed by noncovalent interactions in solution, both in the chemical and in the biological fields. Indeed supramolecular mass spectrometry is far from a routine technique, since a careful optimization of the operating conditions is needed. This latest point will be further on discussed in this paper. For example in the chemical field, ESI-MS is used to determine the stoichiometry and stability constants of synthetic supramolecules with coordinated metal ions [13–16] or maintained by six hydrogen bonds [17]. In the biological field several studies convincingly demonstrate that under carefully controlled operating conditions the weak intermolecular interactions involved in the constitutive formation of specific noncovalent complexes in solution can survive the ESI ionization/desorption process. Thus, many examples of studies of protein/protein, protein/ligand, protein/DNA complexes are described in literature (for some recent reviews, see [18–20]). ESI-MS may have the ability to directly give information on the stoichiometry of the interactions formed in solution with a far better precision than common approaches such as gel shift electrophoresis. Some studies also report that ESI-MS can be used to determine the relative binding affinities in solution [21–25]. More recently, ESI-MS has been proposed as a novel approach to easily probe cooperativity in the binding of a ligand to a multimeric enzyme, with the advantage of giving a direct insight into all the ligation states that are formed in solution as increasing amounts of ligand are added to the enzyme [26]. Another emerging application of ESI-MS is the detection of very large noncovalent multi-protein complexes in their native state (at near physiological pHs) which is a field of heated debate and represents an interesting challenge for mass spectrometrists. The development and commercialization of orthogonal time-of-flight (TOF) analyzers was great progress in the field of supramolecular mass spectrometry, allowing the extend of the experimentally available m/z range from 4000 to 40 000 [27,28]. For instance ESI-MS was used by Green et al. [29] to study large noncovalent multi-protein complexes of globin subassemblies comprising 12 globin chains with molecular weights varying from 204 to 214 kDa. A few years before, with the advent of nanospray, the team of Robinson was able to determine the molecular weights of very large protein complexes such as the chaperone GroEL (a 14-mer assembly of 800 kDa) [30] or ribosomes (850 kDa for the 30S subunit) [31]. Tito et al. [32] obtained a mass spectrum for the intact assembly of the bacteriophage MS2 virus with a measured molecular weight of $2\,484\,700 \pm 25\,200$ Da, which is to date the largest complex observed by ESI-MS. Another example is the detection of well-resolved species of an octamer–trimer (24-mer) of vanillyl-alcohol oxidase at $1\,525\,600 \pm 1000$ Da [33]. More recently Zal et al. [34] reported an ESI-MS analysis of a purified haemocyanin from *Bythograea*

thermydron (deep-sea crab) which was shown to exist as several oligomeric forms, the highest one corresponding to the association of 18 subunits (18-mer) with a molecular weight of 1 354 940 ± 480 Da. Nevertheless, because such ESI-MS experiments need a careful control of all the experimental conditions (desalting procedure, pH, buffer but also mass spectrometry parameters such as the accelerating voltage or the pressure in the interface region of the mass spectrometer) some publications are more pessimistic and avoid the solely use of mass spectrometry for the precise determination of the oligomeric form of a protein [35]. However, as illustrated by several authors [36–38], the applicability of mass spectrometry to investigate noncovalent biological complexes is strongly dependent on the nature of the interactions between the partners: electrostatic interactions are believed to be strongly emphasized while coming from the solution to the gas phase whereas most of the hydrophobic interactions are said to be disrupted by the ESI process. So if hydrophobicity plays a prominent role in the binding, the chance to preserve the edifice during the ESI-MS analysis is highly compromised [39–41]. Thus, the existence of hydrophobic contributions in the stability of a specific noncovalent complex is a prominent factor for the investigation of the given noncovalent edifice by ESI-MS. To date supramolecular mass spectrometry was mainly applied to well-studied or model biological systems. Now we want to extend the use of ESI-MS to characterize unknown or less-well characterized systems, as for example to determine the oligomeric form of *B. subtilis* HPrK/P.

In the present paper, we investigate the oligomeric state of *B. subtilis* HPrK/P and demonstrate that this enzyme is a ∼310 kDa noncovalent homohexamer at pH 6.8. ESI-MS yields a direct control of the formation and the integrity of all oligomeric forms that exist in solution. This is of utmost interest since several oligomeric forms may be significantly present in solution, which may be involved in an unexpected and uncontrolled extend to the binding of substrates. The importance of relevant experimental parameters of the mass spectrometer such as the pressure in the interface region or the accelerating voltage set in the mass spectrometer is illustrated. We also use the potential of biological supramolecular mass spectrometry to monitor the influence of pH variations on the stability of the oligomeric HPrK/P in order to investigate the specificity of the detected interactions.

2. Experimental

2.1. Protein expression and purification

The enzyme HPrK/P (Trx-His_6-S-tag) from *B. subtilis* was expressed in *Escherichia coli* and purified as detailed in [42]. For ESI-MS analysis of HPrK/P, further purification was performed using a HiTrap Q Sepharose High Performance column 1 mL (Amersham Biosciences, Uppsala, Sweden). The HiTrap Q column was equilibrated with 5 column volumes of 25 mM Tris pH 8 followed by 5 column volumes of 25 mM Tris pH 8 containing 1 M KCl, and finally 10 column volumes 25 mM Tris pH 8. The protein solution was applied to the column and different fractions were eluted with 25 mM Tris buffer pH 8, containing increasing concentration of KCl (100, 300, 600 and 1000 mM). The purity of the fractions, after concentration using Ultrafree Centrifugal Filter Unit with molecular weight cut off (MWCO) 10 000 Da (Millipore, Bedford, MA, USA), was confirmed with SDS–PAGE separation using PhastGel (Amersham Biosciences, Uppsala, Sweden) and coomassie staining. The pure fraction(s) were then desalted through a PD-10 column (Amersham Biosciences, Uppsala, Sweden) as described elsewhere [42]. The concentration of HPrK/P was determined spectrophotometrically using the Bio-Rad protein assay (Bio-Rad Laboratories, München, Germany) with Bio-Rad protein assay standard I lyophilised bovine plasma gamma globulin (Bio-Rad Laboratoires, CA, USA) as standard.

2.2. Radioactive kinase assay

A radioactive kinase assay, as described in [42], was used to verify the activity of the enzyme under similar conditions as those used for mass spectrometry.

2.3. Electrospray ionization mass spectrometry

Prior to any mass analysis, the samples were desalted on Centricon PM10 (cut off = 10 000 Da) micro-concentrators (Amicon, Millipore) in 10 mM ammonium acetate (pH 6.8). Ammonium acetate enables native structures of proteins to be preserved and is compatible with ESI-MS analyses. Six dilution/concentration steps were performed at 4 °C and 6000 trs/min.

ESI-MS measurements were first performed on a Quattro II triple quadrupole mass spectrometer (Micromass, Altrincham, UK) with an extended mass range of 8000 *m/z* (second analyzer). Mass spectra were recorded at the exit of the second analyzer, using the first quadrupole in the "rf-only" mode. Further ESI-MS analyses were performed on an electrospray quadrupole time-of-flight mass spectrometer Q-TOF-II fitted with a standard Z-spray source (Micromass, Altrincham, UK) and a *m/z* range extended to 25 000. Again mass spectra were recorded at the exit of the TOF analyzer, the quadrupole was used in the "rf-only" mode.

Purity and homogeneity of the HPrK/P were verified by mass analysis in denaturing conditions: the protein was diluted to 10 pmol/μL in a 1:1 water–acetonitrile mixture (v/v) acidified with 1% formic acid. In these conditions the noncovalent interactions are suppressed, which allows the measurement of the molecular weight of the monomeric subunits with a good precision (better than 0.01%). Mass spectra were recorded in the positive ion mode on the mass range 500–4000 *m/z*, after calibration with horse heart myoglobin diluted to 2 pmol/μL in a 1:1 water–acetonitrile mixture (v/v) acidified with 1% formic acid. Accelerating voltage was set to 40 V and the pressure in the interface region of the mass spectrometer was 2.5 mbar.

In nondenaturing conditions, the mass measurement of the protein was performed in ammonium acetate (10 mM, pH = 6.8) to preserve its native conformation in solution. Samples were diluted to about 20 pmol/μL in the previous buffer and continuously infused into the ESI ion source at a flow rate of 5 μL/min. Great care was exercised so that the noncovalent interactions survive the ionization/desorption process. Particularly in order to preserve the integrity of the noncovalent assemblies and to enhance the sensitivity of the detection, the pressure in the interface between the atmospheric source and the high vacuum region was varied between 2.5 and 7 mbar (the optimal value was found to be 6.5 mbar) by throttling the pumping line. The accelerating voltage applied on the sample cone ranged from 120 to 200 V (optimal value was 200 V) and both source and desolvation temperatures were 80 °C. Clusters of $Cs_{(n+1)}I_n$ (separate injection of a solution of 1 mg/mL CsI in 50% aqueous isopropanol) were used for the calibration of the extended mass range in the high *m/z* region. Mass data were acquired in the positive ion mode on the mass range 2500–12 000 *m/z* for 5 min and smoothed with the Savitzky Golay method. Molecular species were assumed to be represented by series of peaks corresponding to multiply protonated ions. The mass of each species is expressed as a mean of the masses calculated from the series of ions ± standard deviation. Charge state assignments were those that gave minimum standard deviation. The maximum entropy-based software (MaxEnt) was used only to find the approximate mass of each subassembly and hence the charge on each multiply charged peak [43]. Because the MaxEnt software fits symmetrical Gaussian peak shapes to the experimental data, it could not be used to establish the accurate mass, since the peaks were asymmetrical due to adduct formation. The relative abundance of the different species present on ESI mass spectra were measured from their respective peak intensities, assuming that relative intensities displayed by the different species on the ESI mass spectrum faithfully reflect the actual distribution of these species in solution.

3. Results

3.1. Sequence homogeneity and purity verification

In this work, the analysis of the 467 residues *B. subtilis* HPrK/P is reported. The gene was inserted

into a Trx-His_6-S-tag expression vector. The encoded protein was overproduced in *E. coli* and purified as described in the experimental part.

After the previously described desalting and concentration procedures (see "Section 2"), electrospray ionization mass spectrometry analysis in denaturing conditions revealed a highly pure and homogeneous HPrK/P sample. The following molecular weight of 51 700 ± 1.0 Da was measured for the monomer (data not shown), which is in good agreement with the molecular mass calculated for the expected amino acid sequence (51 699.3 Da).

3.2. Nondenaturing ESI-MS analysis of HPrK/P

The first ESI-MS analyses of HPrK/P were performed on a triple quadrupole instrument with a mass range of 8000 *m*/*z* for the second analyzer. The conditions were optimized for the detection of large noncovalent complexes (up to 250 kDa) as described by Rogniaux et al. [36]. However, as the pressure in the interface region of the triple quadrupole could not be increased above 4 mbar, only ions corresponding to monomeric and dimeric forms of HPrK/P were detected (data not shown). In the upper mass range (7000–8000 *m*/*z*) an additional ion series was observed. The weak intensity of these ions and the low signal-to-noise ratio did not allow us to draw any conclusions. That is why we moved to a hybrid quadrupole time-of-flight instrument (Q-TOF-II) that provides a far better precision, an extended mass range (upper-limit: 25 000 *m*/*z*) and the ability to increase the pressure in the interface region to 7 mbar. The relevance of the pressure regulation in the interface region of the mass spectrometer will be further on detailed in the second part of this paper. All mass spectra further presented in this work were obtained on the Q-TOF instrument (see "Section 2").

Fig. 1A shows the ESI-MS mass spectrum of HPrK/P diluted to 20 pmol/μL in a 10 mM ammonium acetate buffer (pH 6.8) obtained under carefully controlled experimental conditions (the accelerating voltage—voltage applied on the sample cone—was set to 120 V and the pressure in the interface region of the mass spectrometer was set to 6.5 mbar, see Fig. 2). Three main ion series were detected: (1) the major set of peaks with a charge state distribution ranging from 38+ to 44+ (the 41+ charge state being the most abundant) was observed in the mass range 7000–8100 *m*/*z* and led to a molecular weight of 310 337 ± 22 Da corresponding to the noncovalent association of six HPrK/P subunits; (2) a second minor ion series corresponding to the 103 404 ± 2 Da dimer with charge states ranging from 21+ to 25+ (centered on the 23+ charge state) was detected in the mass range 4000–5000 *m*/*z*; (3) the third distribution with charge states ranging from 15+ to 17+ corresponded to monomeric subunits detected in the mass range 3000–4000 *m*/*z*. No ions corresponding to either trimeric, tetrameric or pentameric associations of HPrK/P subunits were detected. After MaxEnt deconvolution of the main ions series (mass range *m*/*z* 2500–8100), one main highly homogeneous species with a molecular weight of ∼310 kDa was generated in the 250–400 kDa mass range (see Fig. 1B), corresponding to the noncovalent association of six subunits. Few amounts of monomeric and dimeric HPrK/P were noticed.

The biological activity (see "Section 2") of HPrK/P was checked in a radioactive phosphorylation assay under the same experimental conditions as those used for ESI-MS analysis (10 mM ammonium acetate, pH 6.8): the enzyme was shown to be fully active. It could thus be concluded that ESI-MS is able to give an accurate image of a specific noncovalent hexamer of HPrK/P that pre-exist in solution at pH 6.8.

3.3. Influence of important experimental variables of the mass spectrometer (accelerating voltage and pressure in the interface region) on the detection of the noncovalent HPrK/P hexamer

In order to optimize the operating conditions necessary for the detection of the intact noncovalent hexamer, the influence of both the accelerating voltage (V_c) and the pressure in the interface region of the mass spectrometer (P_i) on the detection of ions corresponding to the hexamer was investigated. Fig. 2 presents a

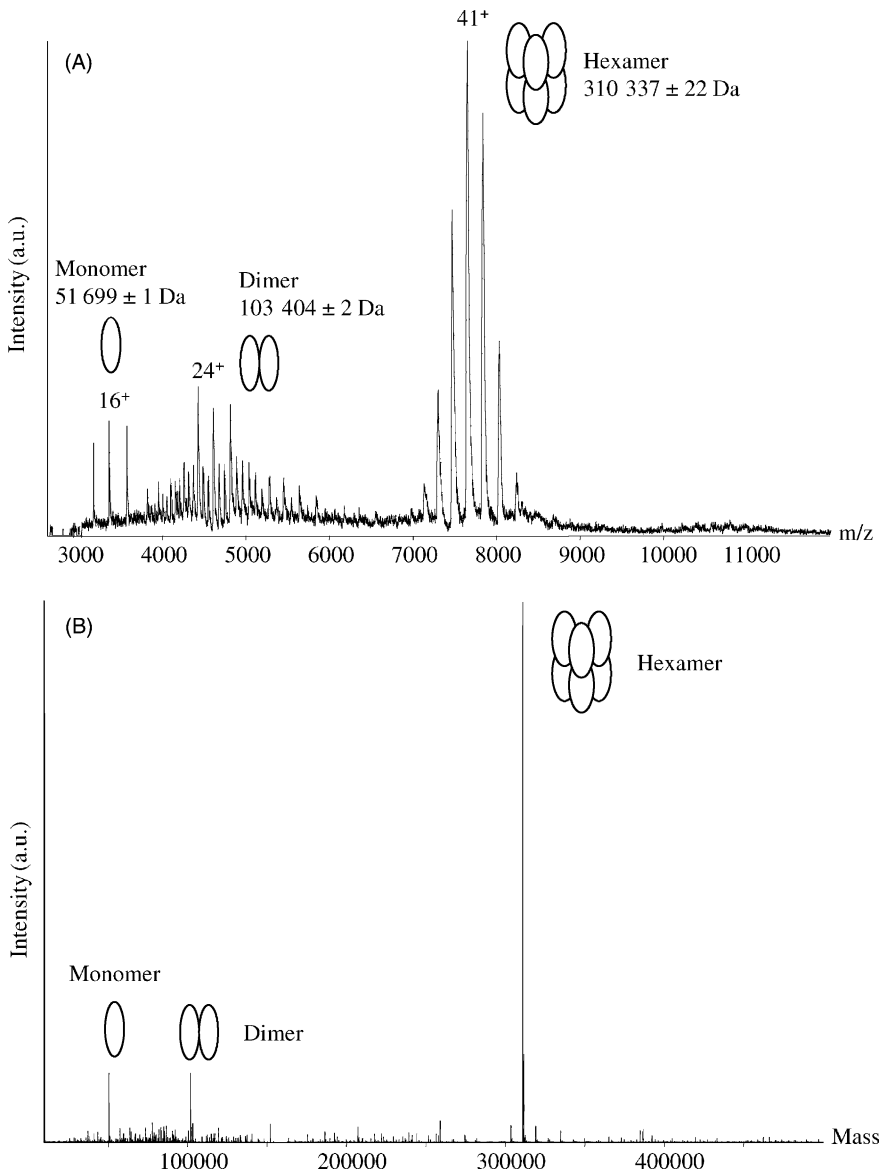

Fig. 1. ESI-MS analysis of HPrK/P noncovalent homohexamer. ESI-MS mass spectra of HPrK/P from *B. subtilis* in nondenaturing conditions. The accelerating voltage V_c was set to 120 V and the pressure in the interface region P_i was 6.5 mbar. The hexameric HPrK/P was diluted to 20 pmol/μL in ammonium acetate 10 mM, pH 6.8. (A) ESI-MS raw data spectrum; (B) ESI-MS spectrum after MaxEnt deconvolution.

schematic view of the interface of the Q-TOF instrument used for this study.

Fig. 3A–C present mass spectra of the enzyme obtained by varying the pressure in the interface region of the mass spectrometer (P_i). At a pressure of 2.5 mbar (pressure normally achieved in the interface of our Q-TOF-II), the main species detected corresponded to the dimeric form of HPrK/P (see Fig. 3A). In the lower *m*/*z* region, few amounts (∼10%) of ions corresponding to the monomer were detected. In the upper *m*/*z* region (above *m*/*z* 6000), ions corresponding to the hexamer (∼20%) were observed. However, it should

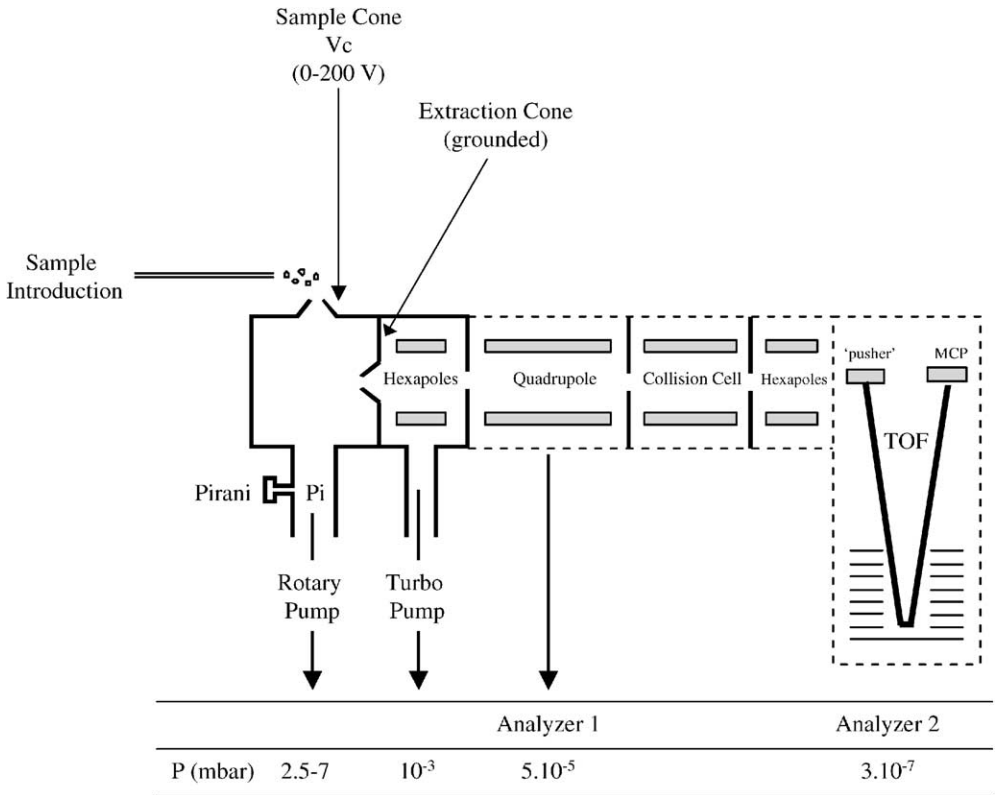

Fig. 2. Schematic view of the interface of the Q-TOF-II instrument (Micromass, Altrincham, UK). The values of the pressures measured at different pumping stages are presented. The voltages applied on relevant lenses are also indicated.

be noticed that the signal-to-noise ratio on this mass spectrum was very low. Increasing this pressure to 4 mbar was achieved by throttling the pumping line (see Fig. 3B) and resulted in a noticeable increase of the signal-to-noise ratio. Monomeric and dimeric HP rK/P were still detected as main components whereas simultaneously the amount of ions corresponding to the hexamer increased (∼30% of the total amount of ions detected). A further increase of the pressure to 6.5 mbar led to dramatic changes on the mass spectrum (see Fig. 3C): ions detected in the high *m*/*z* region and corresponding to the hexamer were detected as major component. In the lower *m*/*z* region only few amounts of monomer and dimer (∼10% each) were observed. From this study it could be concluded that transmission and subsequently detection of intact high *m*/*z* ions were favored at high interface pressures. Varying the pressure in the interface region of the mass spectrometer clearly shows that conclusions about the absence/presence of an oligomer in solution must be drawn with great care since results are dramatically dependent on the experimental conditions.

Fig. 4A and B present ESI-MS mass spectra recorded at different accelerating voltages (V_c—the voltage applied on the sample cone) while P_i was set to the constant value of 6.5 mbar (see Fig. 2). At $V_c = 120$ V, ions corresponding to the monomer, the dimer and the hexamer were detected, hexameric ions being the most abundant ones (Fig. 4A). A progressive increase of V_c to 200 V did not induce drastic changes on the mass spectra (Fig. 4B). However, one additional minor ion series was detected in the mass range 9000–11 000 *m*/*z*. The molecular weight was attributed to the existence of the noncovalent association

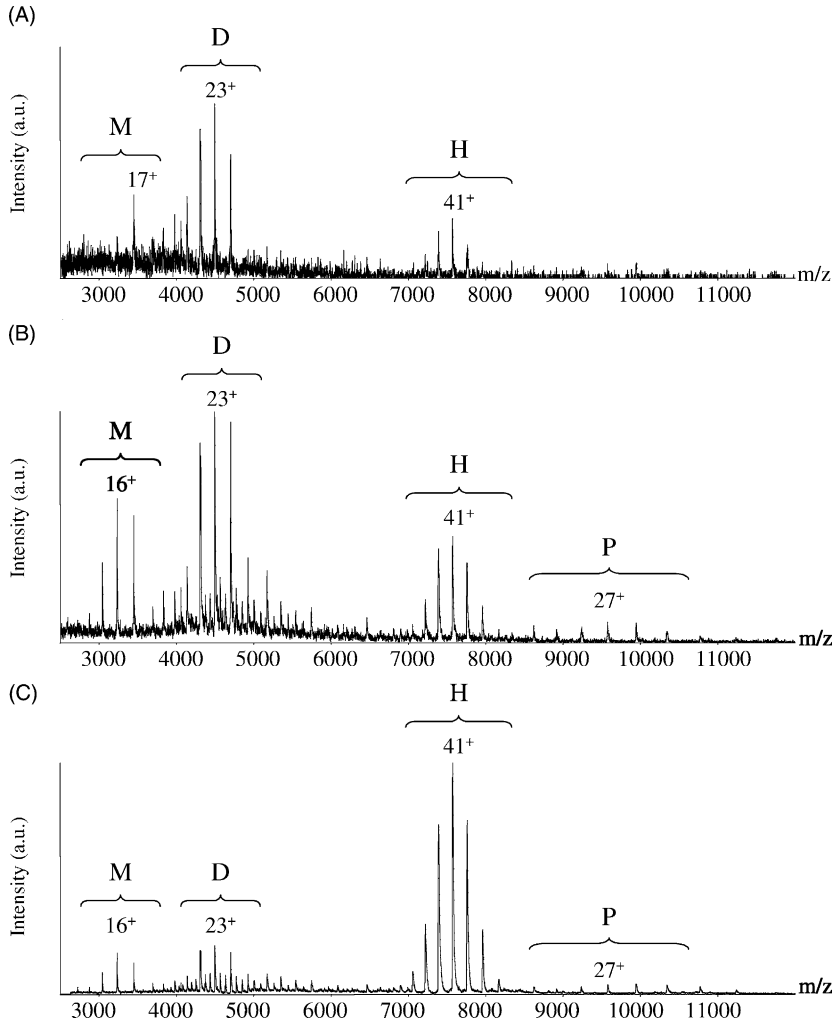

Fig. 3. Influence of the pressure (P_i) in the interface region of the mass spectrometer on the detection of the hexameric HPrK/P. Typical ESI-MS mass spectra recorded at different pressures in the interface (P_i) region of the mass spectrometer. The accelerating voltage V_c was set to 200 V and the hexamer was diluted to 20 pmol/μL in ammonium acetate 10 mM, pH 6.8. (A) $P_i = 2.5$ mbar; (B) $P_i = 4$ mbar; (C) $P_i = 6.5$ mbar. M: monomeric form; D: dimeric form; P: pentameric form; H: hexameric form of HPrK/P.

of five monomeric subunits (5-mer). As those species were only detected at high V_c values, it could be concluded that pentameric ions resulted from a gas-phase dissociation of the hexameric noncovalent complex. Indeed, the detection of specific ions corresponding to pentameric HPrK/P formed in solution would have led to ions appearing in the lower mass range (between 5000 and 7000 *m/z*) and with intermediate average charges per subunit comprised between those of the dimeric and hexameric species. Our results are in agreement with previously published data on collision-induced dissociation of noncovalent complexes [44]: gas-phase collisions are said to lead to asymmetrical dissociations of high molecular weight noncovalent complexes. Thus, collision-induced dissociations of the hexamer (Fig. 4B) lead to pentameric ions (27+) and monomeric ions (16+). However, increasing the accelerating voltage from 120 to 200 V

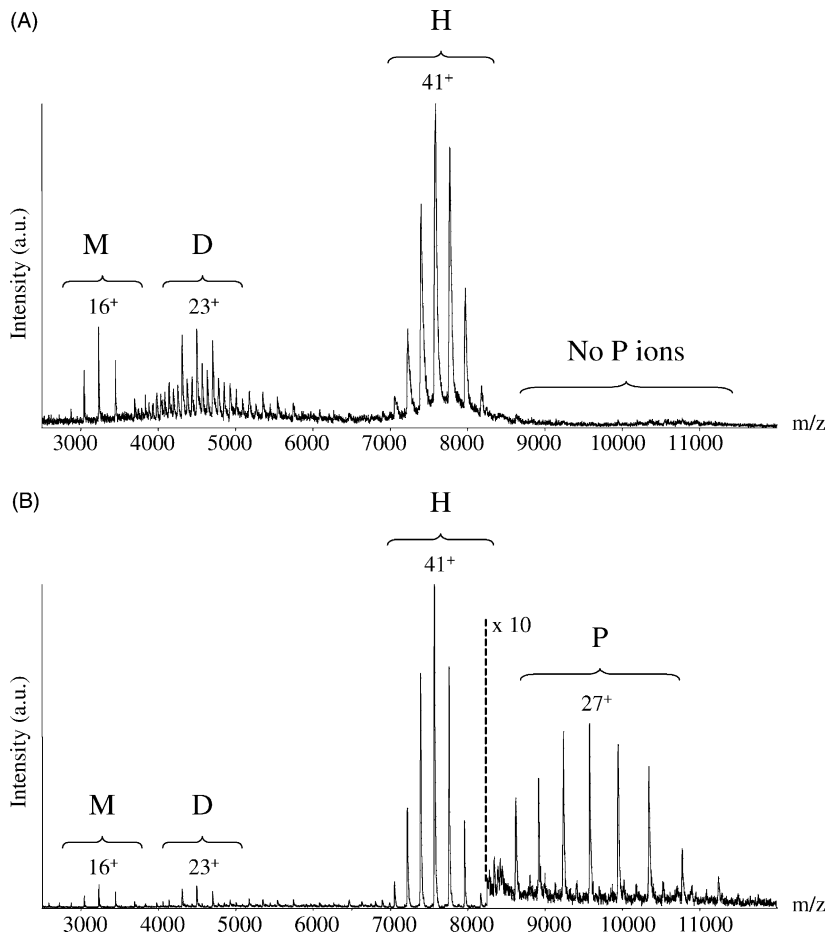

Fig. 4. Influence of accelerating voltage (V_c) on the detection of the hexameric HPrK/P. Typical ESI-MS mass spectra recorded at different accelerating voltages (V_c) in the ion source of the mass spectrometer. P_i was set to 6.5 mbar and the hexamer was diluted 20 pmol/μL in ammonium acetate 10 mM, pH 6.8. (A) $V_c = 120$ V; (B) $V_c = 200$ V. M: monomeric form; D: dimeric form; P: pentameric form; H: hexameric form of HPrK/P.

considerably improved the signal-to-noise ratio of the mass spectra and allowed a far better efficiency of high *m*/*z* ions desolvation. Thus, further experiments were performed at 200 V, knowing that this voltage will induce a slight gas-phase dissociation (<10%) of the hexamer but will considerably enhance desolvation.

3.4. Influence of the pH of the solution on the detection of the HPrK/P hexamer

Another relevant prerequisite for the study of noncovalent complexes by ESI-MS is the ability to demonstrate that ESI-MS detected interactions result from a "structurally-specific" binding and not from any artefactual association. Thus, we studied the influence of pH variations of the ammonium acetate buffer on the stability of the HPrK/P hexamer. ESI-MS experiments were performed at pHs ranging from 4.8 to 9.5. The pH of the 10 mM ammonium acetate buffer was adjusted with diluted ammonia or acetic acid solutions to obtain desired pHs. Fig. 5A–C show the mass spectra obtained at different pHs. Before any ammonia or acetic acid addition, the pH of the ammonium acetate—10 mM buffer was 6.8

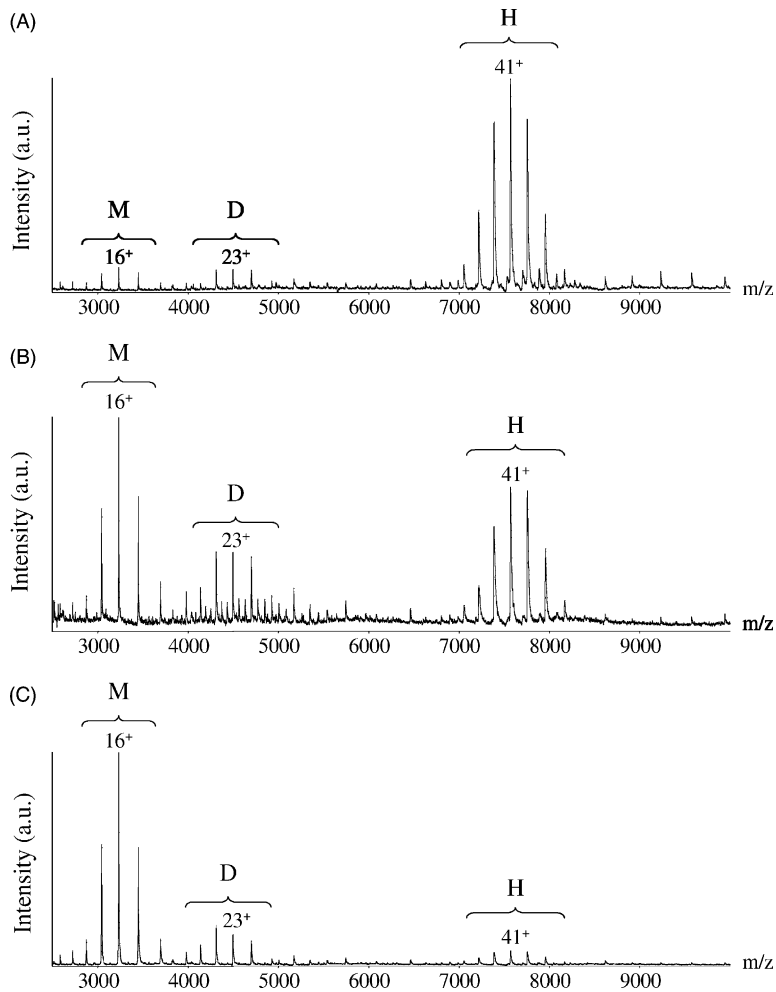

Fig. 5. Influence of pH variations on the stability of the noncovalent HPrK/P hexamer. Typical ESI-MS mass spectra recorded at different pHs for the ammonium acetate buffer. The pH value of the ammonium acetate buffer was adjusted by adding small amounts of ammonia. All mass spectra were recorded at $V_c = 200$ V and $P_i = 6.5$ mbar (the hexamer was diluted to 20 pmol/μL in ammonium acetate 10 mM). (A) pH = 6.8; (B) pH = 8.5; (C) pH = 9.5. M: monomeric form; D: dimeric form; H: hexameric form of HPrK/P.

(see Fig. 5A). As previously described, at pH 6.8, the main component detected corresponded to the HPrK/P hexamer (∼80%). Increasing the pH of the buffer resulted in dramatic changes on mass spectra recorded under strictly the same experimental conditions (in all experiments the accelerating voltage was set to 200 V and the pressure in the interface was 6.5 mbar; the hexamer concentration was estimated to be 20 pmol/μL). At pH 8.5 (see Fig. 5B), the intensity of the ions corresponding to the hexamer (∼35%) decreased whereas the intensities of ions corresponding to both monomer (∼50%) and dimer (∼15%) increased. At pH 9.5 (see Fig. 5C), the main ion series could be related to monomeric (∼70%) and dimeric (∼20%) forms of HPrK/P while only ∼10% of the detected ions corresponded to the hexameric HPrK/P. These observations enabled us to conclude that basic pHs completely destabilize the hexameric structure of HPrK/P into dimeric and monomeric HPrK/P.

Similar experiments were performed by adding increasing amounts of acetic acid to the 10 mM ammonium acetate solution. Unfortunately, the addition of very low amounts of acetic acid induced a complete precipitation and denaturation of the enzyme. After centrifugation, the resulting sample was analyzed by ESI-MS in native conditions and no trace of enzyme was detected. When the precipitated sample was analyzed under denaturing conditions, the molecular weight of the constitutive subunits of the hexamer was measured (data not shown), which strongly suggests that precipitation of the enzyme occurred for low pH values.

These quite simple control experiments allowed us to conclude that under our operating conditions the detection of hexameric HPrK/P can be attributed to "structurally-specific" interactions that preexist in solution.

4. Discussion

4.1. The enzyme HPrK/P in B. subtilis is a noncovalent homohexamer at pH 6.8

The HPrK/P of low-GC Gram-positive bacteria is a serine protein kinase which is involved in the hierarchical control of carbohydrate utilization [9–12,45]. In this work we shed light on the oligomeric form of HPrK/P in *B. subtilis*, expressed in *E. coli* and fused with a Trx-His_6-S-tag in order to facilitate the purification. ESI-MS allowed to unambiguously demonstrate that this enzyme is a homohexamer at pH 6.8. The activity of the HPrK/P was also tested in a radioactive assay under the same conditions as those used for mass spectrometry. Using 10 mM ammonium acetate (pH 6.8) as buffer, the native enzyme was found to be fully active. Classical methods such as size exclusion chromatography or analytical ultracentrifugation often failed in the precise determination of the oligomeric form of HPrK/P. The oligomerization state of the enzyme became a point of heated debate since the last 5 years, when the gene encoding HPrK/P was identified during the *B. subtilis* sequencing program [10,11]. However, data concerning the oligomerization state of HPrK/P from different bacteria are often approximative and confusing. For instance, Brochu and Vadeboncoeur [46] reported that the native HPrK/P in *Streptococcus salivarius* migrates by molecular sieve chromatography as a homogeneous 330 000 Da protein (measured mass of the monomer was 34 440 Da), suggesting that native HPrK/P was a decamer. However, no complementary technique was used to confirm the results obtained by gel filtration chromatography. At the same time, Jault et al. [47] reported that *B. subtilis* HPrK/P, expressed with a His_6-tag (expected molecular weight of the monomer is 36 kDa), formed homo-oligomers constituted most likely of eight subunits. In this study, size exclusion chromatography analyses were performed and revealed one main peak corresponding to a highly oligomeric enzyme of about 260 kDa. Additional sedimentation-equilibrium analytical ultracentrifugation experiments were also conducted and led to a single homogeneous species of average molecular mass 240–320 kDa. The combination of the two techniques suggested that *B. subtilis* HPrK/P was a homo-octamer, although the possibility of a heptamer could not be ruled out. Kravanja et al. [9] also deduced from size exclusion chromatography data that HPrK/P from *Enterococcus faecalis* (mass of the monomer 35 kDa) existed as a dimer, which is surprising regarding the high oligomeric structures proposed for other bacteria. Again no complementary technique was used to confirm this hypothesis. Recently Fieulaine et al. [48] managed to get information on the X-ray three-dimensional structure of the 319 residues catalytic domain of *Lactobacillus casei* HPrK/P, overproduced in *E. coli* and expressed with a His-tag (measured molecular weight of the monomer was 22 654 Da). In solution, both size-exclusion chromatography and equilibrium sedimentation showed that the *L. casei* HPrK/P formed a stable and homogeneous oligomer of ∼140 kDa, which may be consistent with a hexamer. Crystals were also constituted of hexamers which exhibit a dihedral three-fold symmetry, composed of two trimers in two layers, with extensive contacts within trimers and weaker ones between trimers. Thus, it seems quite unambiguous

that *L. casei* HPrK/P forms a hexamer. Our ESI-MS results obtained on *B. subtilis* HPrK/P (with an additional Trx-His_6-S-tag) seem to be in agreement with those obtained by X-ray crystallography for *L. casei* HPrK/P. Even if the tertiary structure of HPrK/P may probably be similar for different bacteria, it is however less certain that the quaternary structure is conserved [48]. However, since crystallographic studies are the only way to have a precise picture of the active site or of the surfaces involved in the interaction, ESI-MS will never be able to replace crystallography. But the crystallization of such large macromolecules is often a limiting step, both time and material consuming compared to ESI-MS. Our ESI-MS results strongly and unambiguously support the fact that *B. subtilis* HPrK/P is a noncovalent homohexamer at pH 6.8 constituted by the association of six subunits, assuming that the tag (Thioredoxine-His_6-tag, Trx-His_6-S-tag) used for the purification of the protein does not interfere with the quaternary structure and the activity of our protein. Comparison of some specific experiments made by different groups using different constructions support this assumption [49]. However, it cannot be ruled out that the "wild-type" enzyme (without tag) may behave in a different way.

The mass measured in nondenaturing conditions is 0.05% higher than the mass predicted from the denatured analysis of HPrK/P. This mass difference is very low compared to other mass measurements made on noncovalent subassemblies [30,32,34] and is not significant given the uncertainty of the measurements in nondenaturing conditions. However, this difference is presumably due to the inclusion of water molecules or small cations that are not present in the denatured monomer. Despite this discrepancy, there is no doubt that HPrK/P exists as a hexamer at pH 6.8.

4.2. Relevance of experimental parameters of the mass spectrometer for the mass measurement of large noncovalent complexes

The potential of ESI-MS to gain information on the oligomeric state of proteins is an interesting field for mass spectrometrists. Several publications report the ability of ESI-MS to record spectra of intact macromolecules [26,29–38]. However, all authors agree with the fact that the detection of noncovalent high molecular weight complexes is not easy at all, since those macromolecules should be driven from the solution to the gas phase without destruction of the weak framework. So the essential prerequisite to the use of ESI-MS for the determination of the oligomeric structure of an enzyme is that the observed peak on mass spectra in vacuo are reliable to species effectively present in solution. Great care in the data acquisition as well as in the interpretation and mass calculation must be taken, since it is quite established that the solution-phase image might be distorted due to several factors during the ESI-MS analysis. In particular during the evaporation of the ions in the gas phase, or during the transfer from the ion source to the analyzer through the interface region of the mass spectrometer.

4.2.1. Influence of the pressure (P_i) in the interface region of the mass spectrometer

We demonstrated in this paper the relevance of gas-phase collisions, which are likely to occur in the interface region of the mass spectrometer, a region of still rather high pressure (1–3 mbar) which may induce the disruption of certain noncovalent assemblies. The detection of the ions corresponding to the HPrK/P hexamer was favored when the pressure in the interface region of the mass spectrometer (P_i) was set to higher values, at least 5 mbar (see Figs. 2 and 3A–C). Indeed P_i, the pressure in the region between the source which is at atmospheric pressure and the first hexapoles, is directly linked to the internal energy communicated to the ions via collisions with residual gaseous molecules present in this part of the mass spectrometer. As P_i is inversely proportional to the distance between two consecutive collisions with ambient gaseous molecules, lower pressures in the interface region (1–3 mbar) imply long distances between two successive collisions. Consequently, gas phase ions have enough time to be "warmed up" and to accumulate internal energy which further results in high

energy and "destructive" collisions. The final consequence may be the dissociation of certain large noncovalent subassemblies even if desolvation of ions may be more efficient. Inversely, increasing the pressure in the interface region gives rise to more frequent but low energy and less destructive collisions after which "thermalized" ions corresponding to large macromolecules are transferred without any destruction to the analyzer. Elevating pressure also leads to a less efficient desolvation of the ions, which is further observed on the ESI mass spectra by significant peak broadening. However, to date there is no clear explanation and no available modelization of the phenomena that happen in the interface region of the mass spectrometer. In fact, the ion transfer from the solution to the gas phase is still not well understood and lots of questions are still open. A proper modelization of the phenomena that take place in the interface region of the mass spectrometer needs to take simultaneously different points into account: existence of a gradient of pressure in the interface region, kinetic of desolvation, variation of the mean free path, local pH changes, loss of hydrophobicity. The group of Standing and coworkers [50] has developed a "collisional damping" interface between atmospheric pressure and high vacuum for an orthogonal ESI-TOF mass spectrometer to try to better understand those interface phenomena. The introduction of hexapoles or a quadrupole in front of the TOF analyzer is believed to "cool" the ions (ions of mass ∼1000 kDa may acquire kinetic energies of ∼1000 eV in the supersonic jet expansion/declustering process). The fact that an increase of the pressure in this interface region enhances the detection efficiency of high *m*/*z* ions has been extensively studied in our group since 10 years [26,36–38]. Several other authors have also reported that elevating the interface pressure enables the detection of large noncovalent subassemblies [30,32–35,50]. The observation that high *m*/*z* ions are more readily detected at high interface pressures may be explained by the collisional cooling effect precisely described by Krutchinsky et al. [50]. Furthermore, we observed that the pressure effect was *m*/*z*-dependent, as effects were observed only for ions above *m*/*z* 4000, which is in agreement with previously reported data from Tahallah et al. [35]. Finally the pressure in the interface region of the mass spectrometer is a crucial parameter for the detection of large noncovalent subassemblies, even if no precise modelization of all the phenomena that take place in this region is to date available.

4.2.2. *Influence of the accelerating voltage (V_c)*

In the same time, varying the accelerating voltage (V_c) induced a change of the initial kinetic energy communicated to the ions in the electrospray source (see Fig. 2). At high accelerating voltages, produced ions have higher initial kinetic energies. Consequently, strong energetic collisions are induced, that may lead to in-source dissociations of weak interactions. To exclude that some species were dissociated upon gas-phase collisions and thus present on the mass spectrum was ruled out by performing experimental measurements at different accelerating voltages: pentameric ions were only detected at high accelerating voltages (from 150 to 200 V) and thus corresponded to collision-induced ions. However, at a fixed P_i value, these measurements showed that the relative ratios of hexameric, dimeric and monomeric ions were not at all modified by gas-phase collisions of increasing energies (see Fig. 4A and B). Decreasing V_c led to a considerable loss in sensitivity due to a nonoptimal transmission of high *m*/*z* ions and to a much less efficient desolvation resulting in a far lower mass accuracy. As desolvation is much more efficient and as the focalization of high *m*/*z* ion is much better at high accelerating voltages, the interpretation of the recorded mass spectra is easier. Consequently, the peak broadening effect previously mentioned for high interface pressures can be reduced by increasing V_c.

Consequently, a compromise between sufficient desolvation, optimal transmission of intact high *m*/*z* ions and nondestructive gas-phase collisions needs to be set to be able to detect specific noncovalent edifices of high molecular weights. A careful optimization of the interface pressure and the accelerating voltage, different for each noncovalent assembly, is necessary to get best results. Thus, performing systematically control

experiments in which both the accelerating voltage and the pressure in the interface region of the mass spectrometer vary are a prerequisite to unambiguously conclude for an ESI-MS detection of specific noncovalent interactions.

4.3. The observed HPrK/P hexamer can be related to a "structurally-specific" noncovalent complex

In noncovalent interactions, the question of specificity of binding is an important issue. One needs to unambigously distinguish between "structurally-specific" noncovalent complexes and between nonspecific noncovalent complexes resulting from any gas-phase or in-solution artefactual association. As precisely detailed by Smith and Light-Wahl [51], several control experiments can be performed in order to assume the detection of specific interactions by ESI-MS. For instance we obtained mass spectra in which the predominant complex was the hexamer by adjusting the interface conditions (P_i and V_c), which is a first good evidence of a specific association in solution. Another type of control experiments consists in studying the complex dissociation when modifications of solution conditions are achieved. In this paper we investigated the influence of changes in pHs (pH values ranging from 6.8 to 9.5, see Fig. 5A–C) on the stability of the HPrK/P hexamer. We demonstrated that under strictly identical experimental conditions an increase towards basic pH values induced the destruction of the hexamer into monomer and dimer. Thus, a change in pH produced a corresponding change in ESI mass spectra. The effect of the pH on the oligomeric state of the enzyme was related to the opposing activities of the bifunctional enzyme HPrK/P [42]. Depending on the environment, the enzyme can phosphorylate and dephosphorylate two proteins (HPr and Crh). Separate experiments evaluating the activity of the bifunctional HPrK/P at different pH values suggest that the phosphatase activity is mainly supported by the hexameric form, whereas the kinase activity may be supported by the dimeric (or maybe also trimeric) form of the enzyme [42]. The combination of all the experiments performed on HPrK/P provided the basis for a high level of confidence for a "structurally-specific" hexamer that exists in nature.

5. Conclusions

In this paper, we used the benefits of ESI-MS to unambiguously determine the oligomeric structure of *B. subtilis* HPrK/P (expressed with a Trx-His_6-S-tag). It was unambiguously demonstrated that this enzyme forms a specific noncovalent homohexamer of ∼310 kDa at pH 6.8. A detailed study of the experimental conditions required to perform high molecular weight mass measurements was presented. Especially dissociation of the noncovalent edifice was induced by subjecting the ions to increasing gas phase collisions either by varying the pressure in the interface region of the mass spectrometer (P_i) or the accelerating voltage (V_c), which were shown to be crucial parameters to preserve the integrity of noncovalent assemblies, to favor the transmission and enhance the sensitivity of the detection of high m/z ions. The fact that pH variations induced strong changes on the ESI mass spectra provided a high level of confidence for a "structurally-specific" hexamer and was correlated more to the phosphatase than kinase activity of the bifunctional enzyme HPrK/P [42].

The approach using ESI-MS that we propose in this paper to investigate the oligomeric forms of a protein presents evident benefits in comparison to classical approaches which are its rapidity, sensitivity, ability to have good indications on most important species present in solution. Moreover, ESI-MS is a low material-consuming technique which is surely a driving force of the approach to help in the determination of the oligomeric state of a protein. However, as previously explained, great care needs to be exercized to be sure that the detected species faithfully rely on the species present in solution. Thus, control experiments will always be needed to conclude about the specificity of the ESI-MS detected interactions.

Acknowledgements

Authors would like to thank Noelle POTIER and Hélène ROGNIAUX for helpful discussions and critical reading of the manuscript. Brian GREEN is warmly acknowledged for stimulating conversations and for technical help in the modification of our instruments. S.S. thanks the CNRS and Lilly for financial support. H.R. gratefully acknowledges a grant from the Swedish Academy of Pharmaceutical Sciences.

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