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Under non-denaturing solvent conditions, the mean charge state of a multiply charged protein ion formed by electrospray is linearly correlated with the macromolecular surface

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This article is dedicated to Professor Jean-Claude Tabet at the occasion of his 60th birthday

Abstract

The charge states of protein ion species generated by electrospray under non-denaturing solvent conditions are strongly dependent on the occurrence of gas phase proton transfer reactions. Thus, by adding basic compounds to an array of model protein solutions, the charged states of multiply charged ions decrease with increasing the gas phase basicity of these additives. The role played by the basic (lysine and arginine) and acidic (aspartic and glutamic acids) amino acid side chains toward the proton exchange processes has been examined by using a series of basic compounds added to the protein solutions. In the present study, no relationship could be established between the presence at the protein surface of basic or acidic residues and the measured charged states. Actually, independently on their amino acid composition, the protein ions show a linear correlation between their mean charge state and their surface considered as a spherical area.

Keywords: Electrospray; Proteins; Mass spectrometry; Non-denaturing conditions; Charge state; Distribution

1. Introduction

Since its discovery, about 20 years ago, electrospray ionization (ESI) [1] combined with mass spectrometry is routinely used for the structure analysis of proteins. Under denaturing solvent conditions, the number of charges z present within a protein ion generated by electrospray appears as roughly proportional to the molecular weight of proteins, thus leading to m/z values comprised in a limited m/z range accessible to most conventional mass spectrometers. However, the multicharging process of proteins can be influenced by many factors such as the number of ionisable groups of the proteins [2], the solvent conditions [3] (pH, solvant, presence of other solutes), charge transfers occurring in the solution and in the gas phase [4] and the operating mode of the ion source and its interface with the mass analyzer (pressure, temperature, applied voltages, etc.) [5]. The charge state distribution could be further affected by the macromolecular conformation [3a].

Under ESI conditions, it is generally considered that the multiply charged ions are obtained, in the positive ion mode, by protonation of the basic amino acid side chains (arginine, lysine, terminal-amine group and in a lesser extent histidine) and, for the negative ions, by deprotonation of the acidic residues (aspartic and glutamic acids, terminal COOH group and eventually tyrosine) [2]. Although it could be tempting to relate an increase or decrease of the mean charge state to the presence of acidic or basic side chains, respectively deprotonated and protonated in a solution phase at neutral pH, the pK_a values of these residues do not explain by themselves the generation of negative ions from low pH solutions or of positive (protonated) species at pH values higher than the pK_a of the potential basic sites [5d]. A contribution of gas phase processes for the formation of multiply charged ions was thus proposed by Le Blanc et al. Their study of singly and doubly charged peptides provided evidence that the abundance of these cations was not correlated with the solution pH but with the proton affinity of the involved

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bases [6]. The gas phase basicity (GB) of the solvent components should thus be considered as a relevant factor if gas phase proton transfers between protein and solvent ions are expected.

In the gas phase, exposure of protein polycations to strong bases leads indeed to proton transfer reactions [7]. A reduction of the charge state is observed and the ion peaks are shifted to higher *m*/*z* values. A better separation between the ion peaks improves the accuracy of the protein mass measurement and can help to resolve mixtures of protein species of close masses.

In their recent paper, Kebarle and coworkers demonstrated that the ammonium cation was responsible for the protonation in the gas phase of the basic side chains present at the protein surface and that a good correlation could be observed between the experimental *z* values and those deduced from the charged residue model (CRM) [8]. On the basis of the latter model, Lemaire et al. showed that the number of charges of electrosprayed proteins under non-denaturing solvent conditions was stringly reduced when a triethylammonium bicarbonate buffer (TEAB) was used in place of the more currently used ammonium salts [9]. This effect was rationalized in terms of gas phase proton transfer reactions between the basic amino acid side chains and the protonated basic additives of different gas phase basicities (arginine: 987 kJ mol^{-1}, triethylamine: 925 kJ mol^{-1}, lysine: 916 kJ mol^{-1}, histidine: 891 kJ mol^{-1}, ammonia: 808 kJ mol^{-1}) [10]. The authors postulated that a majority of arginines, lysines and histidines were protonated in the experiments carried out with ammonium ions whereas the protonation reactions occurring with the triethylammonium cations involved preferentially the arginine side chains. In such a hypothesis, the charge states measured by ESI mass spectrometry of proteins under non-denaturing solvent conditions should provide information on the tridimensional structure of proteins in the gas phase.

Several groups have been interested by an eventual relationship between the amino acid composition of proteins and their charge states. Smith et al. published a list of peptides and proteins in which a good agreement between the maximum charge states and the number of basic amino acids present in the sequences was observed [11]. However, for some molecules, the correlation failed [11]. It was thus postulated that vicinal basic residues interacting with a common proton could explain a charge number lower than the expected value [4b]. A similar behavior was noticed in the negative ion mode for myoglobin, the mass spectrum not corresponding to its composition in acidic residues [5d]. In our own group, we shown recently that two model proteins of similar molecular weight but of very different composition in arginine and lysine residues led unexpectedly to identical charge states and charge state distributions in the presence of triethylammonium cations [12]. This result was contradictory with our previous hypothesis on the role of the gas phase basicities of the ammonium versus triethylammonium cations with regard to those of the basic amino acid side chains [9]. In order to rationalize this discrepancy, we have examined the effect of the addition of a series of organic bases to aqueous solutions of model proteins on their charge states. These proteins were chosen in function of their molecular weight, of their content in basic and acidic residues, and of their isoelectric point. Some parameters such as the gas phase basicity of the basic additives or the number of basic and acidic residues of the proteins have been submitted to a detailed examination.

2. Experimental

2.1. Materials

Bovine pancreatic insulin, bovine aprotinin, hen egg lysozyme, horse heart myoglobin, trypsin inhibitor (soybean), human carbonic anhydrases I and II, bovine carbonic anhydrase II and bovine serum albumin were purchased from Sigma (Saint-Quentin Fallavier, France). Ammonia (B1), ethylamine (B2), isopropylamine (B3), diethylamine (B4), diisopropylamine (B5), triethylamine (B6), tripropylamine (B7), tributylamine (B8), 1,3-tetramethylpropanediamine (B9), 1,4-tetramethylbutanediamine (B10), were purchased from Sigma and used without any further purification.

2.2. Mass spectrometry

Experiments were performed by using a Zabspec/T mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization source [13]. They were carried out at a skimmer voltage (V_s) of 4 kV (or 2 kV for *m*/*z* values above 10,000) and the sampling cone potential (V_{sc}), controlled by the operator, was adjusted to attain $V_{sc} - V_s$ values comprised between 0 and 240 V. The source temperature was held at 100 °C. The mass spectrometer was scanned over the *m*/*z* range of interest and the mass scale was calibrated by injecting a solution of cesium iodide (Aldrich, Saint-Quentin Fallavier, France). Samples were delivered into the electrospray ion source by means of a syringe pump PHD 2000 (Harvard Apparatus, Les Ulis, France) at a flow rate of 10 μl min^{-1}. Instrumental parameters such as applied voltages (needle, sampling cone, skimmer, ring electrode) were kept at constant values in order not to interfere with the effect of bases on the observed charged states.

The average charge state of the ion peak distribution was calculated as follows:

$$z_{av} = \frac{S\, nI_n}{S\, I_n}$$

where I_n is relative intensity of the peak corresponding to the charge state *n*. In the presence of basic compounds, no significant adduct ion peaks were observed. The z_{av} values thus correspond to multiprotonated protein ions.

2.3. Sample preparation

Protein solutions were desalted by using 3 or 10 kDa cutoff ultrafiltration cartridges (YM3 and YM10 membrane, Amicon-Millipore) with a Jouan BR4i centrifuge (6000 × *g*, 4 °C) prior to mass spectrometric analyses. The 100 μmol l^{-1} (H_2O) protein samples were washed with 300 ml of bases. For the four washings, concentrations of basic solutions were 100, 50, 50 and 20 mmol l^{-1}. The rotation speed of the centrifuge was kept at 7400 rpm for 30 or 60 min according to the studied protein. Desalted proteins whose final concentrations were 20 μmol l^{-1} in 20 mM basic solutions were introduced in the source of the mass spectrometer.

3. Results and discussion

Under non-denaturing solvent conditions, the mean charge state of all investigated proteins is considerably affected by the presence of the basic compounds added to the sample solutions. The mean charge state decreases indeed with an increase of the gas phase basicity of the basic additives (Table 1 and Fig. 1). Thus, as suggested by Felitsyn

Table 1
Charge state of the major ion peak of the listed proteins electrosprayed in 20 mM aqueous solutions of bases B1–B10

Proteins	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
Insulin	4+	3+	4+	3+	3+	3+	3+	3+	2+	2+
Aprotinin	5+	4+	4+	4+	3+	3+	4+	3+	3+	3+
Lysozyme	8+	7+	7+	5+	5+	5+	5+	4+	4+	4+
Myoglobin	8+	7+	7+	5+	6+	5+	6+	5+	4+	4+
Trypsin inhibitor	9+	7+	8+	7+	6+	5+	5+	5+	5+	4+
Human carbonic anhydrase I	10+	9+	9+	8+	7+	6+	5+	6+	6+	5+
Bovine carbonic anhydrase II	10+	9+	9+	8+	7+	6+	6+	6+	6+	5+
Human carbonic anhydrase II	11+	9+	10+	8+	8+	7+	7+	6+	6+	5+
Bovine serum albumin	17+	16+	16+	13+	–[a]	11+	11+	–[a]	9+	8+

[a] No reliable electrospray mass spectrum of BSA was obtained with bases B5 and B8.

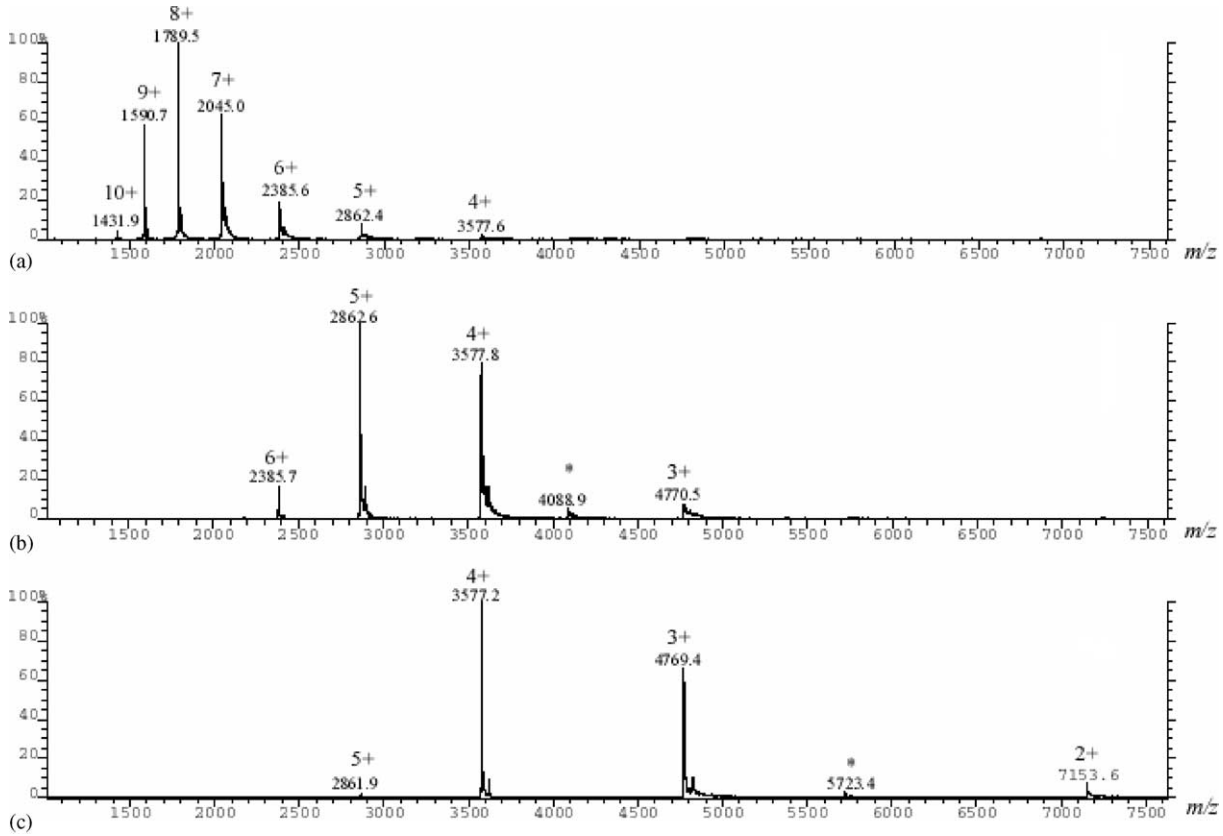

Fig. 1. Electrospray mass spectra of lysozyme at a 20 μmol concentration in aqueous solutions of 20 mM ammonia (B1, a), triethylamine (B6, b) and 1,4-tetramethylbutanediamine (B10, c).

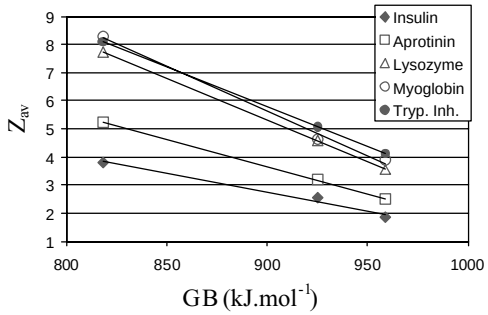

Fig. 2. Averaged charge states of insulin, aprotinin, lysozyme, myoglobin and trypsin inhibitor (20 μmol l^{-1}) in 20 mmol l^{-1} aqueous solution of ammonia (B1), triethylamine (B6) and 1,4-tetramethylbutanediamine (B10) in function of the gas phase basicity of these additives.

et al. [8], protonation reactions could occur between protonated basic additives and some amino acid side chains of proteins in the late desolvation stage in the sampling system. By contrast, the electrospray mass spectra did not show any relationship between the solution pH and the protein charge states. This result is in agreement with those of Wang and Cole who underlined that the protonation rate of a protein under ESI conditions depends only weakly on the solution pH [14].

The correlation between the GB values of the added bases and the charge number of protein ions is illustrated by Fig. 2 which is limited, for clarity, to five proteins (insulin, aprotinin, lysozyme, myoglobin and trypsin inhibitor) and the three aformentioned bases (B1, B6, B10).

It is interesting to note that the GB values led to better correlations with the charge states than the proton affinities of the bases (not shown). A similar observation was made previously by Ogorzalek Loo et al. in a study of gas phase proton transfer reactions involving multiply charged proteins with a modified electrospray ionization atmosphere–vacuum interface [4c].

The gas phase basicity of the additives being directly correlated with the measured charge states of proteins ions, it was necessary to investigate the influence of the polar amino acid side chains toward the multicharging process of proteins under non-denaturing solvent conditions.

For most of the protein solutions used in this work, the pH value was lower than the pK_a of the basic amino acids, in particular arginine (12.48) and lysine (10.54). These residues are thus expected to be generally protonated in the solution phase before the protein ion transfer into the gas phase. Under such conditions, the acidic residues (aspartic and glutamic acids) should be in their deprotonated (carboxylate) form [15]. In view of the potential interest of such a hypothesis for structure investigation of native proteins, we tried to find a relationship between the number of basic sites (*B*) and acidic residues (*A*) of the proteins under investigation and their mean charge state measured by electrospray mass spectrometry. In order to exemplify these results, we will limit the discussion to the five smallest proteins involved in the present study (insulin, aprotinin, lysozyme, myoglobin, and trypsin inhibitor, Table 2).

Table 2
Number of basic (*B*) and acidic sites (*A*) of the listed proteins and the corresponding averaged charge states *z* measured in their electrospray mass spectra recorded in a 20 mM aqueous solution of ammonia

Proteins	*B*	*A*	*B* − *A*	z_{av}
Insulin	5	5	0	3.82
Aprotinine	11	5	6	5.22
Lysosyme	19	10	9	7.74
Myoglobine	33	22	11	8.28
Trypsin inhibitor	22	31	−9	8.11

As expected, the mean charge number increases with the number of basic residues (*B*) but a similar trend can be observed with the acidic groups (*A*). If we consider that the charge state corresponds to the difference between the number of protonated and deprotonated residues within the resulting gas phase ion, it appears clearly that the generation of multiply charged ions from these proteins could involved in a similar extent both the protonation of basic residues (Eq. (1)) and the neutralization of acidic groups (Eq. (2)).

$$\text{Protein-(Lys, Arg)} \cdots \text{H}^+ \cdots B \rightarrow \text{Protein-(Lys, Arg)H}^+ + B \quad (1)$$

$$\text{Protein-(Asp, Glu)-COO}^- \cdots \text{H}^+ \cdots B \rightarrow \text{Protein-(Asp, Glu)COOH} + B \quad (2)$$

In these two equations, *B* corresponds to the basic compound added to the protein solution.

Considering that deprotonated carboxylic groups could be present in protein ions together with protonated basic residues, it seemed more relevant to examine the difference between the number of basic and acidic residues (*B* − *A*) rather than each of them separately. Fig. 3 shows the mean charge state of each protein (z_{av}) measured in the presence of four different bases (B1, B4, B6 and B10) in function of the difference of basic and acidic sites (*B* − *A*).

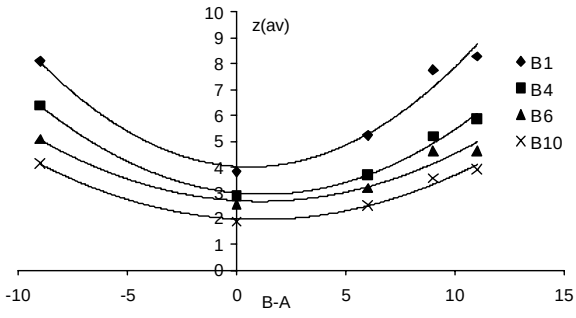

Fig. 3. Averaged charge state of insulin, aprotinin, lysozyme, myoglobin and trypsin inhibitor (20 μmol l^{-1}) in 20 mmol l^{-1} aqueous solutions of ammonia (B1), diethylamine (B4), triethylamine (B6) and 1,4-tetramethylbutanediamine (B10) in function of the difference between the number of basic sites (*B*: lysine, histidine, arginine, terminal NH_2 group) and acidic sites (*A*: glutamic and aspartic acids, terminal COOH group) of the proteins.

It is significant that the trypsin inhibitor, which contains an excess of nine acidic sites, and myoglobin with an excess of 11 basic residues, lead to very close mean charged states in all the basic solutions used. This result underlines the role played by the neutralization of the negative charges in the formation of positively multiply charged protein ions as reported previously [16]. This process could rationalize either the high charge state of the trypsin inhibitor or the charge number increase with the number of acidic sites in the proteins. Considering also that the reactivity of basic and acidic residues is influenced by the presence of neighboring functional groups in the gas phase protein 3D structure, it is difficult to deduce directly the mean number of protonated basic and deprotonated acidic sites within a multiply charged protein from its amino acid composition. As pointed out by Felitsyn et al., for most proteins, the observed charge state z_{obs} corresponds to the value calculated from the CRM and is lower than the number of strongly basic sites in the protein structure [8].

From Fig. 3, it can be observed that the minimum charge state was attained by insulin for which $B - A$ equals zero and that the highest charge states were obtained for proteins containing an excess of basic or acidic sites. However, insulin possesses the lowest molecular mass of the studied proteins and, similarly, it is obvious that the similar charge states of trypsin inhibitor and myoglobin could also be related to their similar masses (19,979 and 17,567 Da, respectively). It was thus necessary to investigate in what extent the molecular mass of the proteins could play a role on their charge states after electrospray ionization.

The relevance of the molecular weight as a factor influencing the charge state of proteins is obvious if we consider that the number of acidic and basic residues is expected to increase with the size of the macromolecule. An empirical formula relating the molecular mass of proteins and the mean charge states was established previously as $\ln(z_{av}) = A\ln(M) + B$, where M was the protein mass [9,17]. However, this relation remained to be rationalized in terms of protein structure and gas phase protein reactivity. Our approach was based on two main assumptions: (i) under non-denaturing solvent conditions, the protein ions are formed from their folded conformation [3], (ii) the gas phase proton transfers involved in the multicharging process occur at the protein surface. It follows that the protein surface should be the relevant parameter for evaluating the protonation rate under non-denaturing electrospray conditions, rather than the molecular weight of proteins. Considering, in that way, that protein ions can assigned roughly spherical structures of identical density (ρ), it is possible to calculate their surface from their molecular mass (M). Provided that the volume (V) of a sphere of diameter D and density ρ is related to the mass by the relation $V = M/\rho$ the molecular mass (M) is given by

$$M(D, \rho) = \left(\frac{\pi D^3}{6}\right)\rho N_0 \quad (3)$$

where N_0 is Avogadro's number. The surface S of a sphere being equal to pD^2, the relationship between mass and surface is

$$M(D, \rho) = \left(\frac{\pi (S/p)^{3/2}}{6}\right)\rho N_0 \quad (4)$$

The surface values of proteins derived from their molecular weights by applying Eq. (4) are given in Table 3. For this calculation, we used the density value of $0.8\,g\,cm^{-3}$ proposed by Kaufman et al. [18]. In this report on the gas phase electrophoretic mobility molecular analysis of proteins, the authors assumed a spherical shape for the singly charged ions. In this model, an "effective density" within the protein volume was considered, a value of $0.8\,g\,cm^{-3}$ providing the best match for most of the data points concerning globular proteins. Considering that the density in multiply charged proteins could be lower than that of singly charged ions because of eventual coulombic repulsions we preferred this value to that of $1\,g\,cm^{-3}$ used by Fernandez de la Mora for calculating the radius of proteins from their molecular mass [19].

Reporting in a graph the mean charge states versus the calculated surfaces allows to obtain linear relations between

Table 3
Molecular weights and calculated surfaces of the listed proteins and the corresponding averaged charge states z measured in their electrospray mass spectra recorded in 20 mM aqueous solutions of B1, B4, B6 and B10

Proteins	MW (Da)	Surface (10^3 Å^2)	z_{av} (B1)	z_{av} (B4)	z_{av} (B6)	z_{av} (B10)
Insulin	5,735[a]	2.52	3.82	2.88	2.55	1.88
Aprotinin	6,512[a]	2.74	5.22	3.69	3.18	2.51
Lysozyme	14,304[a]	4.63	7.74	5.19	4.61	3.55
Myoglobin	17,567[a]	5.31	8.28	5.85	4.62	3.91
Trypsin inhibitor	19,979[a]	5.79	8.1	6.39	5.08	4.13
Human carbonic anhydrase I	28,843[a]	7.40	9.84	7.65	6.16	5.24
Bovin carbonic anhydrase I	29,061[a]	7.44	7.38	7.77	6.40	5.45
Human carbonic anhydrase II	29,242[a]	7.47	8.22	7.63	6.19	5.18
Bovin albumin	66,813[a]	12.96	16.37	8.22	10.99	8.59
Threonine deaminase (dimer)	119,698[b]	19.11	24.15	–	–	–
Threonine deaminase (tetramer)	239,450[b]	30.34	35.00	–	–	–

[a] Calculated mass from the protein sequence.
[b] Measured mass.

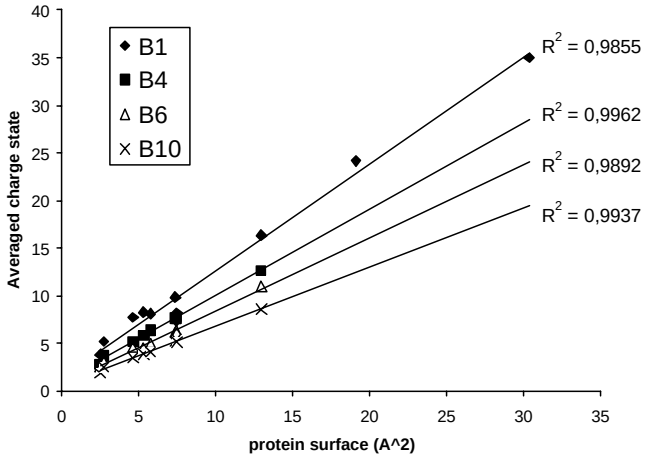

Fig. 4. Averaged charge states of the proteins listed in Table 3 (20 μmol l^{-1}) in 20 mmol l^{-1} aqueous solutions of ammonia (B1), diethylamine (B4), triethylamine (B6) and 1,4-tetramethylbutanediamine (B10) in function of the protein surfaces calculated from their molecular mass by applying Eq. (4).

these two parameters. In Fig. 4, the data are shown for the same four representative bases as above (ammonia-B1, DEA-B4, TEA-B6, TMBDA-B10). In the case of ammonia, two additional points were obtained from the mass analysis of the enzyme threonine deaminase in its dimeric and tetrameric forms [20]. This protein was subjected to analyses without any relationship with the present work. Because of the low amount of sample available, it was not possible to record the spectra in the presence of other bases.

As expected, the slopes of the curves decrease with increasing the gas phase basicity of the basic additives. With the noticeable exception of the carbonic anhydrase proteins electrosprayed in the presence of ammonia, each measured charge state differs by less than one charge from the value indicated by the regression lines. The very close charge states of these three proteins in the other basic solutions explain the observed increase of the correlation coefficients. Thus, the number of charges present in the ions formed by the proteins of our series under non-denaturing conditions is linearly correlated with the molecular surface considered as a sphere. It is particularly interesting to note, as observed by many authors in case of oligomeric proteins, that the charge state of the threonine deaminase tetramer is less than twice that of the dimer. The two corresponding points in Fig. 4 are aligned with those of the other proteins. That indicates that oligomeric proteins follow the same relationship between charge state and molecular surface as the monomeric proteins.

At the present time, the curves shown in Fig. 4 are routinely used in our laboratory to predict the number of charges of protein samples submitted to MS analysis. In each case, the experimental charge state falls within ±1 charge range with regard to the expected value.

From these experiments, it results that no relationship could be established between the amino acid composition of the studied proteins and their charge states measured in the electrospray mass spectra recorded under non-denaturing conditions. The desolvation energy and rate, the occurrence of intramolecular coulombic repulsion, the involvement of other reactive sites than strongly basic or acidic residues (such as the peptidic backbones) play also a significant role in the multicharging process of proteins interacting with strongly basic molecules. The present study needs to be completed by a similar work on multiply charged negative ions under the same solvent conditions.

4. Conclusion

The multiply charged ions generated by electrospray from proteins under non-denaturing conditions can be assigned a spherical structure. The charge state seems to depend mainly on two parameters, i.e., the protein surface, with which it is linearly correlated, and the gas phase basicity of the basic compounds added to the aqueous solution. These results underline the role of the gas phase interactions between the protein molecules and the solvant constituents. However, the difference between the number of basic or acidic amino acids in the sequence has no visible effect on the charge state distribution of these ions and no direct effect of the intrinsic gas phase basicity of these amino acids, generally localized at the protein surfaces, could be evidenced by the present study. Further investigations are thus needed to understand the detailed mechanism of the multicharging process of proteins electrosprayed under non-denaturing solvant conditions.

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