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The Influence of Strongly Acidic Groups on the Protonation of Peptides in Electrospray MS

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In order to obtain experimental data on the question of compensation of positive charges by anionic groups in multiply charged ions of polyfunctional molecules in electrospray MS, several pairs of peptides with the same basic structure but differentiated by one or two strongly acidic groups (phosphate or sulfonate) were investigated. It was found that depending on the density of basic centers and the strength of acidic groups present, the observed changes upon introduction of acidic groups ranged from complete elimination of the most highly charged state to absence of any difference. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

There is much evidence in the literature that multiply charged positive ions of peptides, generated by electrospray ionization (ESI), are not partially neutralized by carboxylate groups under the commonly chosen conditions of optimum sensitivity, i.e. acidic pH and moderate declustering.[1,2]

This evidence is seen most clearly with tryptic peptides. Owing to the specific requirements of the enzyme, these peptides carry either lysine or arginine as C-terminal residue and therefore have at least two basic centers. If they contain histidines, the number of basic sites increases according to the number of these residues, but since histidine residues are relatively rare in nature, the number of basic sites in tryptic peptides rarely exceeds three. In contrast with this situation, the number of acidic groups varies between one, the C-terminal and several groups, depending on the number of aspartic and glutamic acid residues present. The isoelectric point of such peptides therefore varies considerably and may well be below 7.

When examining tryptic peptides by positive ESI-MS, a distribution of charge states which has a maximum at 2+ or 3+ and only a few per cent at 1+ is mostly observed.[1,2] This charge state distribution is found independently of the number of acidic groups present. Obviously, if the C-terminal group or any of the other acidic groups were dissociated, the number of charges should be reduced and a tryptic peptide without additional acidic or basic amino acids should be singly charged because one of its two basic sites should be neutralized by the C-terminus. This is not observed at all, or at most to the small extent to which the singly charged species appears.

With other peptides the same observations are made. As a rule, the number of basic residues determines the number of charges in positive ESI-MS, whereas the number of acidic residues has no influence.[1,2]

Is this rule surprising? Siu and co-workers studied the protonation of proteins and reached the conclusion that the ions which are observed in ESI-MS are essentially preformed in solution, at least under acidic conditions.[3] We want to restrict the present discussion to acidic conditions, thus excluding the as yet puzzling results obtained with protonation in basic solutions.[4,5] Ashton *et al.* have criticized Siu's proposal on the simple ground that in some proteins the maximum number of charges observed exceeds the number of basic residues in the protein and therefore cannot possibly reflect the status of the protein in solution.[6] These authors cast even more doubt on the validity of Siu's postulate by emphasizing the well-known fact that the declustering conditions and other factors strongly influence the charge state distribution. Examining this controversy with regard to peptides, one must conclude that Siu's view appears to reflect qualitatively the experimental results, even though modifications according to Ashton's criticism must be added, e.g. some charging of the backbone during the spraying or some decharging during the declustering process.

In solutions composed of 1N acetic acid and acetonitrile in 1/1 ratio the pH is approximately 2.6, which means that the acetic acid is dissociated in the range of 0.1% (The influence of the aprotic, neutral and polar acetonitrile is considered to be insignificant.) The acidity constants of the acidic groups in peptides, i.e. those of aspartic acid, glutamic acid and the C-terminus, are approximately the same as that of acetic acid, in contrast with the dissociation constants of free amino acids where the pK of the acids is much lower owing to the proximity of the protonated amino group.[7] Therefore it

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must be assumed that the acidic groups in a peptide are only dissociated to the same small extent as the acetic acid in the same solution, i.e. much below 1%. Thus their intramolecular neutralizing potential is negligible when viewed in the context of the total charge distribution of the peptide.

On the other hand, the p*K* of the amino groups in a peptide, lying between 9 and 10, assures their virtually complete protonation at the pH of 1N acetic acid.

Given the fact that in 1N acetic acid, according to the analysis just given, all basic sites must be protonated and all acidic sites are undissociated, why is the protonation observed experimentally in ESI often incomplete? The answer is that the increasing protonation of the peptide leads to increasing repulsion of additional positive charges, or in other words, to a progressive decrease in the proton affinity of the free amino groups in increasingly protonated peptides.[3,6] In a group of closely packed basic residues the repulsion becomes strong enough to reduce the proton affinity of an amino group to a value below that of the solvent, so that the solvent competes successfully for protons and thus prevents complete protonation of the peptide.

In order to support this concept, it appeared interesting to examine modified peptides in which acidic groups with lower p*K* values than that of the carboxyl group are present. It must be expected that a p*K* far below that of acetic acid leads to dissociated groups, resulting in zwitterions and perhaps in intramolecular salt bridges and thus to reduced apparent charging of the peptides.

Phosphates with a first p*K* between 1.0 and 1.5[8] and sulfates or sulfonates with a $pK < 1$ appeared most suitable for these studies.

From a research project unrelated to the present question,[9] a number of pairs of peptides were available to us which contained tyrosine or threonine residues free and phosphorylated. By comparing the behavior of these pairs, the influence of the acidity change can be studied.

For the study of the sulfate no such ideal substrate was available. Therefore we decided to generate pairs of closely related structures by oxidizing cysteine-containing peptides.[10,11] These pairs provided us with the means for a comparison of the very weakly acidic sulfhydryl and the strongly acidic sulfonate group in otherwise equal structures. Such pairs have been used before for mass spectrometric studies with a different objective, namely the elucidation of the influence of strongly acidic residues on the CAD fragmentation of tryptic peptides after FAB ionization.[12] Very recently the fragmentation of specifically synthesized pairs of peptides containing two arginine and one or two cysteine residues was studied in the original and in the oxidized form.[13] With regard to the present question, these authors noted in a case where two arginines were balanced by the cysteines that the base peak was due to doubly protonated molecules in both the original and the oxidized form. Apparently the neutralizing action of the strongly acidic residues opened the way for additional protonation of the less basic sites in the molecule, as indicated by the strongly increased fragmentation of the backbone in the more acidic counterpart of the pair.[13]

In the following we want to present a number of cases of multiply charged ions where the acidic groups manifest themselves by changing the charge state distribution in a number of different ways.

EXPERIMENTAL

Synthetic peptides available from studies in pharmaceutical projects[9] were selected according to their suitability for the intended purpose. They had to contain at least two basic sites and, besides the C-terminal carboxyl group, one, two or three tyrosines, threonines or cysteines. The phosphoric esters were prepared according to established procedures on a preparative scale.[9] The cysteines or cystines were oxidized with performic acid on a microscale.[10,11] Ten microliters of a mixture of 10 volumes of H_2O_2 (30%) and 90 volumes of formic acid were added to 100 μl of a 1 mM solution of the peptide in acetonitrile and 1N acetic acid (1/1). After several minutes at room temperature an aliquot of 10 μl was withdrawn, diluted at least 10-fold with a mixture of acetonitrile and 1N acetic acid (1/1) and analyzed. In cases where the reaction was still not complete, as indicated by peaks due to the parent peptide, the analysis was repeated after longer reaction times.

Except where noted, the conditions for all analyses were kept constant. The samples were dissolved in a mixture of acetonitrile and 1N acetic acid (1/1, v/v) in a concentration around 10 pmol μl^{-1}. Five microliters of the solutions were flow injected into a stream of 20 μl min^{-1} of acetonitrile/water (1/1) and introduced into the mass spectrometer by pneumatically assisted electrospray (ion spray) at 5 kV. The declustering voltage was 60 V. The mass spectrometer was of type API III, Perkin–Elmer SCIEX, Thornhill, Canada, controlled by a Macintosh computer. The acquisition and data-processing software was from the same company.

RESULTS AND DISCUSSION

Sulfonates

Figure 1 shows the ESI mass spectrum of a peptide with 19 residues, one of them a cysteine residue as oxidizable group. The peptide contains four basic residues, one arginine and three lysines, and two acidic residues. As discussed in the Introduction, the carboxylic groups are of no relevance in the present context. Fortuitously this peptide was contaminated with a product of an incomplete synthesis, lacking the N-terminal cysteine from an otherwise identical structure. The mass spectrum of the mixture of the two original peptides shows a very similar charge distribution for both of them, i.e. the one with the cysteine residue as well as the one without. Both have five basic centers. The 5+ peaks are quite intense, while the 4+ peaks are the base peaks. The intensity drops to almost zero at the 2+ peaks. Apparently the cysteine residue has no influence on the charging pattern. As expected, it behaves like an unpolar

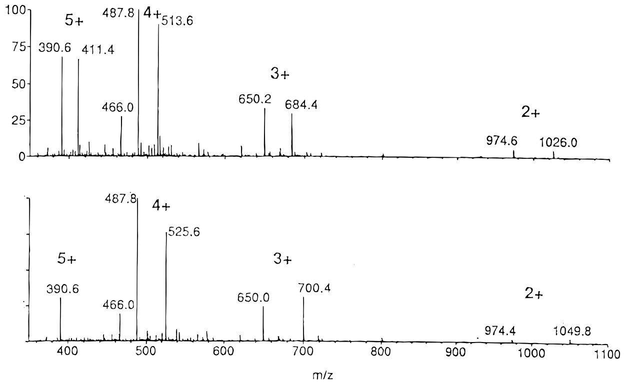

Figure 1. Electrospray mass spectra of cysteine-containing peptide **C**SYGA**KK**GNTLEEP**K**GT**R**L (top) and its cysteic acid derivative (bottom) from solutions at equal pH (basic sites emphasized). The corresponding des-cys-peptide is present in both panels.

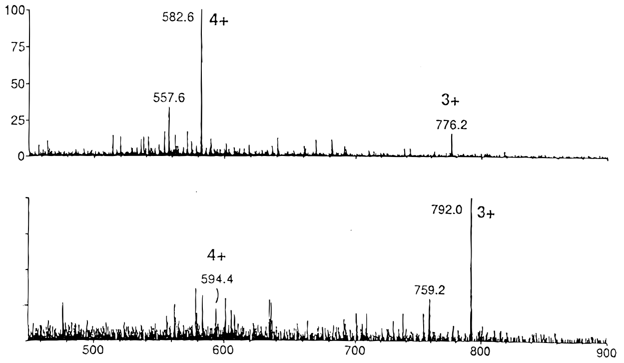

Figure 2. Electrospray mass spectra of cysteinyl-containing peptide Ac-**K**GVSS**R**SYVGCI**K**NLEIS**R**ST-NH_2 (top) and its cysteic acid derivative (bottom) from solutions at equal pH.

residue. Oxidation of the sample changes the pattern of the cys-peptide, which now contains a cysteic acid residue, thus a sulfonic acid group. As shown in the bottom panel of Fig. 1, the 5+ peak of this peptide, expected at *m/z* 421, has disappeared completely from the spectrum, while the unchanged peak at *m/z* 390.6 still represents the 5+ peak of the cysteine-lacking peptide. The explanation for this result is in line with Siu's postulate. The peptide is still fully protonated, but the cysteic acid is dissociated and thus neutralizes one positive charge, causing the disappearance of the 5+ peak.

Apparently there is no significant influence on the intensity of the ions with lower charges. Naively one might assume that the whole peak pattern would be shifted downward by one charge, the base peak moving from 4+ down to 3+, but obviously this does not occur. Excluding the 5+ peak, the patterns of the peptide are remarkably similar before and after oxidation. From this observation it must be concluded that the cysteic acid residue is indeed dissociated as anticipated[12,13] and thus neutralizes one protonated group. The rest of the protonation proceeds as if two neutral residues were present instead of a pair consisting of a basic and an acidic residue.

It must be emphasized that in this case a portion of the molecules of the original peptides achieves protonation of all its five basic sites. In view of the fact that only five out of 19 amino acids are basic, complete charging of a portion of the molecules is not surprising. A charge density of 5+ on a sequence of 19 residues, resulting in peaks below *m/z* 400, is frequently observed in peptides.

A related example, shown in Fig. 2, is a peptide with four basic sites on 21 amino acid residues. In this case the charging also reaches the maximum level at the basic sites, 4+. In the corresponding sulfonate, again generated by oxidation of the cysteine residue to cysteic acid, the maximum charge is essentially reduced by one to 3+, as could be anticipated from the previous example. Nevertheless, a small peak corresponding to the 4+ state remains at *m/z* 594.4. If full dissociation of the sulfonate is assumed, then the 4+ state must be attributed to an additional protonation of the backbone. That such behavior can indeed attain great importance is shown by the following example.

Figure 3 shows the spectrum of a peptide with relatively few basic sites: three on 21 amino acid residues. Essentially complete protonation is observed, i.e. this peptide forms mainly triply charged ions. A very small portion is quadruply charged, apparently representing ions which achieve protonation of one amide nitrogen, not totally surprising in view of the low content of amino groups. In this case the oxidized product behaves distinctly differently from the peptides examined before. The triply charged ions still constitute the base peak, although its relative intensity is somewhat reduced with respect to that of the doubly charged ions. It must be assumed that the neutralizing effect of the sulfonate reduces repulsion of the positive charges to such an extent that amide protonation becomes prominent. Again it must be pointed out that the *m/z* value of 786.6 of this triply charged ion is still rather high compared with the *m/z* values of the highest charged ions of peptides in general.

The next two cases to be discussed belong to the class of moderately basic peptides, three basic centers on 15 amino acids (Fig. 4) and two on six (Fig. 5), where full amine protonation is achieved but no amide protonation is observed in the original peptides. The protonation of the corresponding sulfonates is reduced by one, from 3+ to 2+ and from 2+ to 1+ respectively.

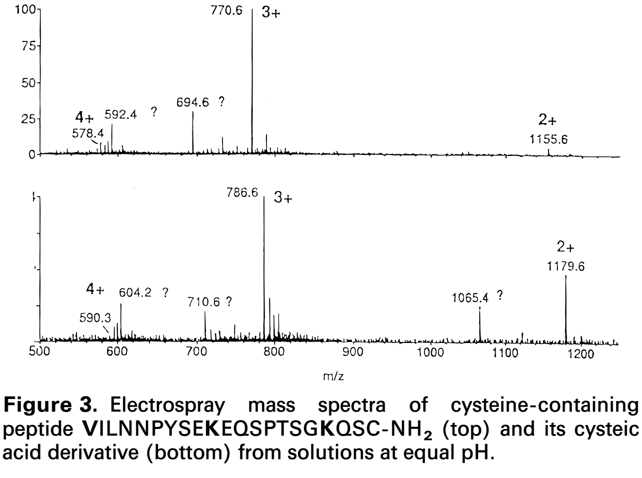

Figure 3. Electrospray mass spectra of cysteine-containing peptide **V**ILNNPYSE**K**EQSPTSG**K**QSC-NH_2 (top) and its cysteic acid derivative (bottom) from solutions at equal pH.

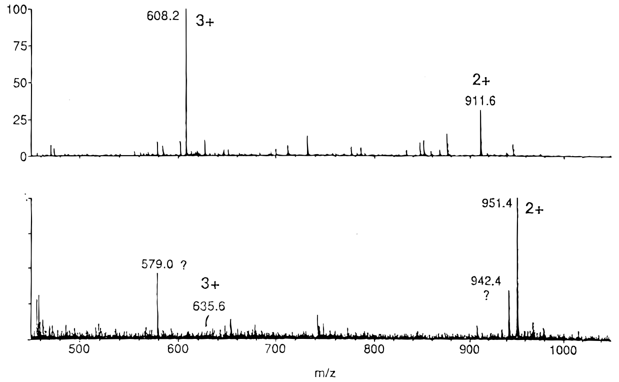

Figure 4. Electrospray mass spectra of cysteine-containing peptide RDSPEDFVYQFKGMC (top) and its cysteic acid derivative (bottom) from solutions at equal pH.

Thus in these two cases the sulfonate obviously neutralizes one of the positive charges and no compensation by protonation of the backbone occurs. These peptides show special features because the first contains a methionine and the second a tryptophan residue, both of which are also oxidizable under the conditions of the cysteine oxidation. However, since neither of the two yields acidic oxidation products, they only cause mass shifts, but no changes in the charging. In Fig. 5 it is seen that three peaks, *m*/*z* 831.8, 847.6 and 863.6, have been obtained from the tryptophan, differing in mass by twice 16 Da.[11] These three peaks represent three different oxidation products of tryptophan, whose structures have not been elucidated but which are expected to be non-acidic.[11] All three show the same charging pattern. The same is of course true for the oxidation product of methionine, a sulfone (Fig. 4), which has also no influence on the charging.

Two or more sulfonates in a peptide should be able to neutralize two or more protonated sites.[12,13] The B chain of insulin which contains two cysteine residues is a case in point [Fig. 6(a)]. The concentration of basic sites in the insulin B chain is relatively low, five in 30 amino acids, so maximum charging is achieved to a considerable extent. Apparently no charging of an amide bond occurs, although judging only from the high *m*/*z* of the highest charged peak, *m*/*z* 681, this might have been expected. An intermediate result is obtained from the oxidized form which contains two cysteic residues. The maximum charge is reduced by one instead of two, from 5+ to 4+. In addition, the total intensity pattern is shifted one charge down, changing the base peak from 4+ to 3+.

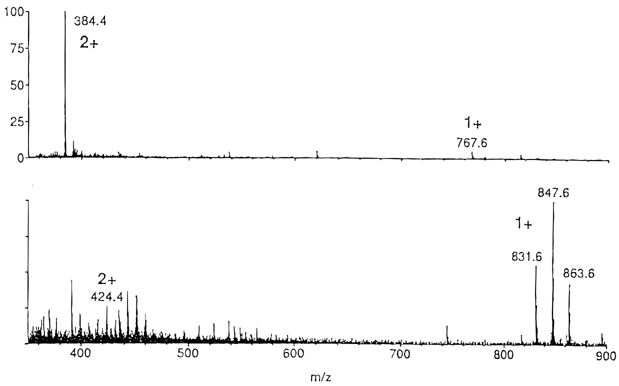

Figure 5. Electrospray mass spectra of cysteine-containing peptide FWICAK (top) and its cysteic acid derivative (bottom) from solutions at equal pH.

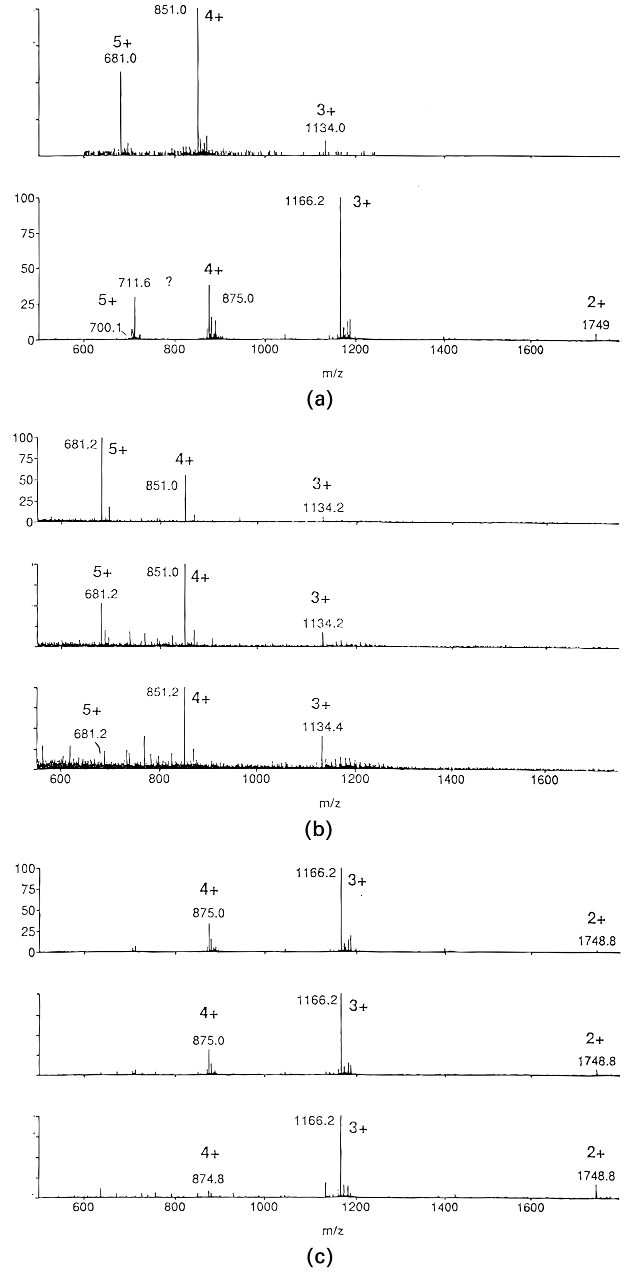

Figure 6. (a) Electrospray mass spectra of B chain of insulin with two cysteine residues, FVNQHLCGSHLVEALYLVC-GERGFFYTPKA (top) and its bis-cysteic acid derivative (bottom), from solutions at equal pH. (b, c) As (a), but spectra taken at three different declustering voltages (40, 80 and 100 V).

Again this result must be explained by assuming amide protonation which has become possible through the reduction of repulsion of the positive charges. The shift in the intensity pattern can be attributed to the less complete protonation of an amide compared with all the amines present.

It is well known that the charging pattern is influenced by the declustering energy that is used.[6] Up to this point this parameter has been kept constant at 60 V.

The case of the insulin B chain appeared to be a suitable example for the examination of the influence of this parameter on the neutralizing effect of negative charges.

In Fig. 6(b) the change in the charging pattern of the original, unoxidized insulin B chain is compared at orifice voltages of 40, 80 and 100 V. While the patterns at 40 and 80 V are similar to that observed at 60 V [Fig. 6(a) top], the stronger declustering at 100 V causes a strong reduction of the charging. The highest charged state, 5+, is eliminated and the whole pattern is shifted to lower charges.

The influence of the declustering energy on the oxidized product is closely analogous to that on the original peptide, as shown in Fig. 6(c). At 40 and 80 V the patterns are similar to that at 60 V. At 100 V an equally strong reduction of charging is observed as with the original peptide: almost complete disappearance of the highest charge, 4+, and a shift of the whole pattern to lower values.

This means that the difference between the cys-peptide and its oxidized derivative observed at 60 V is equally present at 100 V declustering energy: The two sulfonates cause the reduction of charging by one unit [Fig. 6(b), bottom and Fig. 6(c), bottom].

Summarizing the observations leads to the conclusion that the sulfonates are fully dissociated in protonated peptide ions, as would be expected from their p*K* value (Table 1). They therefore neutralize invariably an equal number of positive charges. Whether this reduction in charging can be observed in the mass spectrum depends essentially on the density of positive charges in the ions.

Two cases can be differentiated.

If, owing to scarcity of amino groups, the density of charges in the original peptide is *low* after complete protonation of all basic sites, then protonation of an amide bond upon oxidation of a cysteine residue becomes likely, because the overall repulsion of the positive charges is diminished by the newly created negative charge. The result then is that the same highest charged ion is observed in comparable abundance [Figs 3 and 6(a)].

If the density of amino groups is relatively *high* but complete protonation is still reached in the original peptide, then it is likely that a sulfonate group reduces the maximum charge by one unit (Figs 4 and 5).

Phosphates

As pointed out in the Introduction, the first p*K* value of phosphoric acid monoesters is between 1.0 and 1.5[8] and thus much higher than that of sulfonates.

Table 1. Structures of original peptides and their acidic derivatives with maximum charged ions[a]

	A	B	C
Figure 1	**C**SYGA**KK**GNTLEEP**K**GT**R**L	5/19	5+ (70%)
	(SO_3H)		4+ (100%)
Figure 2	Ac-**K**GVSS**R**SYVGCI**K**NLEIS**R**ST-NH_2	4/21	4+ (100%)
	(SO_3H)		4+ (15%)
Figure 3	**V**ILNNPYSE**K**EQSPTSG**K**QSC-NH_2	3/21	3+ (100%)
	(SO_3H)		3+ (100%)
Figure 4	**R**DSPEDFVYQF**K**GMC	3/15	3+ (100%)
	(SO_3H)		2+ (100%)
Figure 5	**F**WICA**K**	2/6	2+ (100%)
	(SO_3H)		1+ (100%)
Figure 6	**F**VNQ**H**LCGS**H**LVEALYLVCGE**R**GFFYTP**K**A	5/30	5+ (60%)
	(SO_3H) (SO_3H)		4+ (30%)
Figure 7	**TR**DNIYETDYY**RK**	4/13	4+ (60%) 4+ (20%) 4+ (10%)
	$(PO_3H_2)_2$		
Figure 8	**C**IV**RKR**TL**RR**	6/10	5+ (50%)
	(PO_3H_2)		5+ (25%)
Figure 9	**K**NIV**KK**GYLL**KK**G**K**	7/14	5+ (80%)
	(PO_3H_2)		5+ (60%)
Figure 10	**K**EPQYEEIPIYL	2/12	2+ (100%)
	(PO_3H_2)		2+ (100%)

[a] A, sequence of peptide, original and (derivatized), with **basic sites** emphasized; B, number of basic sites/number of residues; C, highest observed charge (percentage of base peak).

Nevertheless, in a solution of 1N acetic acid (or 0.1N formic acid) it must be assumed that such groups are dissociated to an extent exceeding 90%, the exact degree of dissociation depending on the structural environment.

The following experiments were designed, like those described above with the sulfonates, to elucidate the degree of overall charge reduction by phosphorylation of peptides.

Figure 7 shows the mass spectra of peptides with a relatively high density of basic sites, four on 13 amino acid residues. The original unphosphorylated peptide (Fig. 7, top) achieves full protonation of its basic sites with rather high intensity, the peak at *m/z* 406.7 corresponding to 4+ charges.

This spectrum is compared with those of its monophosphate, Y11-PO_3H_2 (Fig. 7, center) and diphosphate, Y10,Y11-$(PO_3H_2)_2$ (Fig. 7, bottom). The comparison shows that the phosphate groups do not totally eliminate the peak with highest charge, 4+, but they reduce its intensity markedly. One must conclude that the phosphate group is only partially dissociated and thus has only a modest effect on the positive charging. When the same experiment is run in 1N formic acid instead of 1N acetic acid, the result is almost identical (data not shown). In view of the fact that formic acid is slightly stronger (p*K* 4.5) than acetic acid (p*K* 4.9), this result confirms the known fact that slight changes in the pH of the solution do not strongly influence the charging of a peptide.

Figures 8 and 9 show peptides with a very high density of basic sites, six on 10 and seven on 14 amino acid residues respectively. Owing to their high base density, both peptides lack ions with full protonation, 5+ being the highest charge in both cases. Comparing the original, unphosphorylated and the phosphorylated peptides, it is seen that the patterns are essentially unchanged. The conclusion can only be that the degree of dissociation of the phosphate is fully compensated by additional protonation of the fifth and the sixth basic site respectively.

In contrast with the previous examples, the next peptide is one with a very *low* base density, two basic sites on 12 amino acid residues (Fig. 10). The two sites available are quasifully protonated, but somewhat surprisingly no amide protonation occurs despite the fact that the basic amino acid is N-terminal and a proline residue occurs near the other end of the molecule. Phosphorylation leaves the pattern practically unchanged, except for the expected mass shift. This is somewhat puzzling, because it means that now the dissociated portion of the phosphate is fully compensated by amide protonation. Under these circumstances it is not possible to estimate the degree of dissociation of the phosphate.

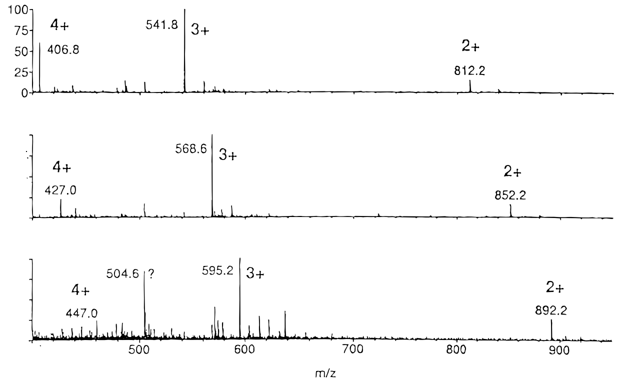

Figure 7. Electrospray mass spectra of tyrosine-containing peptide **TR**DNIYETDYY**RK** (top), its (Y10)-phosphate derivative (middle) and its (Y10,Y11)-bis-phosphate derivative (bottom) from solutions at equal pH.

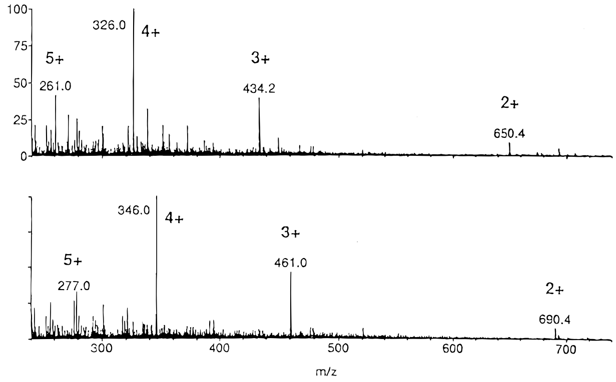

Figure 8. Electrospray mass spectra of threonine-containing peptide **C**IV**RKR**TL**RR** (top) and its (T)-phosphate derivative (bottom) from solutions at equal pH.

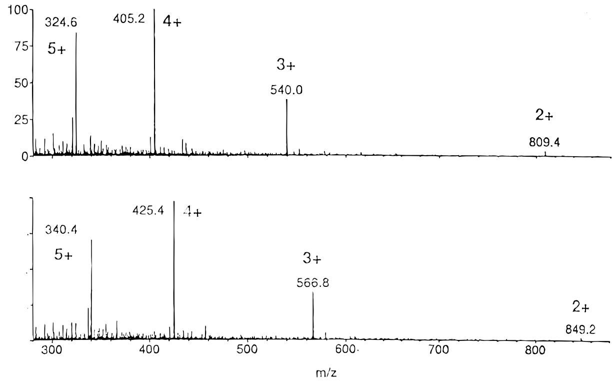

Figure 9. Electrospray mass spectra of tyrosine-containing peptide **K**NIV**KK**GYLL**KK**G**K** (top) and its Y-phosphate derivative (bottom) from solutions at equal pH.

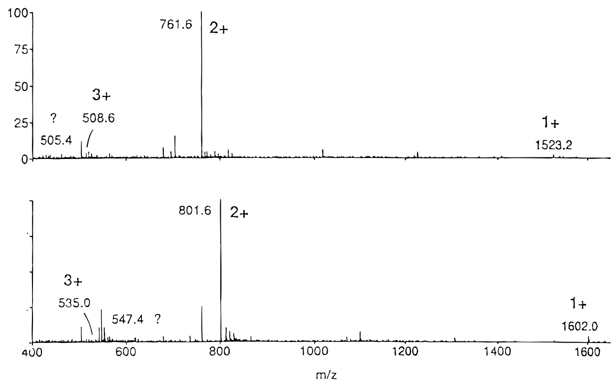

Figure 10. Electrospray mass spectra of tyrosine-containing peptide **K**EPQYEEIPIYL (top) and its Y-phosphate derivative (bottom) from solutions at equal pH.

Summarizing the results obtained from the phosphorylated peptides (Table 1) leads to the same conclusions as have been reached with the sulfonates, except that dissociation of the phosphates is probably incomplete in most cases. This is demonstrated most conspicuously in the case of the completely protonated peptide with relatively high base density (Fig. 7), where the highest charged state is only partially eliminated by one phosphate and even not entirely by a second phosphate. Incomplete dissociation of the phosphate, combined with compensating protonation on still available free amino groups and even protonation of amide nitrogens (Fig. 10), usually generates patterns of the phosphorylated peptides which are almost indistinguishable from those of the original, unphosphorylated analogs.

The unexpectedly low degree of dissociation observed in the phosphates leads us to assume that a substantial decrease in the pH accompanies the electrospray process, presumably owing to the oxidation reactions which take place in the electrolysis of the solution.[14]

CONCLUSIONS

The incorporation of strongly acidic groups into a peptide shows only a limited influence on its protonation in electrospray mass spectra obtained in the positive ionization mode and in weakly acidic solution. Three categories of peptides can be distinguished with respect to their charging pattern.

The first category comprises peptides which have a low density of bases in their amino acid sequence and therefore have an intense peak for fully protonated amino groups (and possibly even some additional protonation of the amides in the backbone). Anionic groups in such peptides tend to reduce the total positive ionization but cause increased amide protonation and thereby induce partial compensation for the neutralized charge.

The second category consists of peptides with a density of basic amino acids which is just sufficient to generate some ions with fully protonated amino groups but no amide protonation. In this case the introduction of a negative charge causes the disappearance of the highest charged state. Sulfonates cause complete disappearance owing to their complete dissociation, whereas with phosphates the charge reduction appears incomplete, presumably owing to only partial dissociation under the conditions employed.

Finally there is the category of peptides with a high density of bases in their sequence, where the protonation is incomplete, showing no peak for ions which have all amino groups protonated. In this case a negative group has no significant effect on the charging pattern. Apparently the negative charge is simply compensated by protonation of an additional amino group.

No attempts to quantitate these results have been made. A much larger assembly of pairs of peptides with and without negatively charged groups would probably be needed to provide a basis for such a quantitation.[15]

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REFERENCES

1. R. D. Smith, J. A. Loo, C. G. Edmonds, C. J. Barinaga and H. R. Udseth, *Anal. Chem.* **62**, 882 (1990).
2. R. D. Smith, J. A. Loo, R. R. Ogorzalek, M. Busman and H. R. Udseth, *Mass Spectrom. Rev.* **10**, 359 (1992).
3. R. Guevremont, K. W. M. Siu, J. C. Y. Le Blanc and S. S. Berman, *J. Am. Soc. Mass Spectrom.* **3**, 216 (1992).
4. M. A. Kelly, M. M. Vestling, C. C. Fenselau and P. B. Smith, *Org. Mass Spectrom.* **27**, 1143 (1992).
5. J. C. Y. Le Blanc, J. Wang, R. Guevremont and K. W. M. Siu, *Org. Mass Spectrom.* **29**, 587 (1994).
6. D. S. Ashton, C. R. Beddell, D. J. Cooper, B. N. Green and R. W. A. Oliver, *Org. Mass Spectrom.* **28**, 721 (1992).
7. J. L. Wu and C. B. Lebrilla, *J. Am. Soc. Mass Spectrom.* **6**, 91 (1995).
8. W. D. Kumler and J. J. Eiler, *J. Am. Chem. Soc.* **65**, 2355 (1943).
9. E. A. Kitas, R. Knorr, A. Trzeciak and W. Bannwarth, *Helv. Chim. Acta* **74**, 1314 (1991).
10. A. Fontana and C. Toniolo, *Prog. Chem. Nat. Prod.* **33**, 324 (1976).
11. Y. Sun and D. L. Smith, *Anal Biochem.* **172**, 130 (1988).
12. O. Burlet, Ch.-Y. Yang and S. Gaskell, *J. Am. Soc. Mass Spectrom.* **3**, 337 (1992).
13. K. A. Cox, S. Gaskell, M. Morris and A. Whiting, *J. Am. Soc. Mass Spectrom.* **7**, 522 (1996).
14. P. Kebarle and L. Tang, *Anal. Chem.* **65**, 972A (1993).
15. K. M. Downard and K. Biemann, *Int. J. Mass Spectrom. Ion Process.* **148**, 191 (1995).