pH Regulated Formation of Side Products in the Reductive Amination Approach for Differential Labeling of Peptides in Relative

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Research Article

pH-regulated formation of side products in the reductive amination approach for differential labeling of peptides in relative quantitative experiments

Among the most common stable-isotope labeling strategies, the reaction of formaldehyde with peptides in the presence of $NaCNBH_3$ features many attractive aspects that are conducive to its employment in quantitation experiments in proteomics. Reductive amination, with formaldehyde and d(2)-formaldehyde, is reported to be a fast, easy, and specific reaction, undoubtedly inexpensive if compared with commercially available kits for differential isotope coding. Acetaldehyde and d(4)-acetaldehyde could be employed as well without a substantial increase in terms of cost, and should provide a wider spacing between the differentially tagged peptides in the mass spectrum. Nevertheless, only a single paper reports about a diethylation approach for quantitation. We undertook a systematic analytical investigation on the reductive amination of some standard peptides pointing out the occasional occurrence of side reactions in dependence of pH or reagents order of addition, particularly observing the formation of cyclic adducts ascribable to rearrangements involving the generated Schiff-base and all the nucleophilic sites of its chemical environment. We also tried to evaluate how much this side-products amount may impair isotope coded relative quantitation.

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Diethylation / Dimethylation / Quantitative analysis / Reductive amination / Stable isotope labeling
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1 Introduction

One of the most crucial issues and growing areas of modern proteomics is the relative quantification of the differential expression of proteins in two or more samples representing various conditions of biological systems. The stable isotope labeling techniques developed in the last two decades for relative quantitation experiments space from in vivo metabolic incorporation of heavy isotopes, or isotopically labeled aminoacids [1, 2], to in vitro experiments with chemical reagents [3–6]. Many recent reviews give a comprehensive picture of the current tendency for relative quantitative proteomics with stable isotope labeling [7–11].

Reductive amination with formaldehyde and $NaCNBH_3$ is a simple reaction that involves all the free amino groups of peptides, namely N-termini and lysine residues, replacing hydrogens with two methyl groups [12]. A differential isotope labeling can be achieved by employing d(0)-formaldehyde and d(2)-formaldehyde obtaining a mass increment of 28 and 32 Da, respectively, for each derivatized reactive site. Acetaldehyde and d(4)-acetaldehyde are still relatively inexpensive reagents and can be used as diethylating agents, by the same approach, providing a wider separation, in terms of mass units, between the differentially labeled peptides [13].

The chemistry of both formaldehyde and acetaldehyde in biological systems features interesting aspects for proteomics investigations. Formaldehyde has been widely used

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Abbreviations: ACTH, adrenocorticothropic hormone fragment 18–39; **EM**, exact mass; **FA**, formic acid; **HCCA**, α-cyano-4-hydroxycinnamic acid; **2MEGA**, dimethylation guanidination; **NaOAc**, sodium acetate; **TDP**, renin substrate tetradecapeptide human; **TEAB**, triethylammonium bicarbonate; **TEAOAc**, tetraethylammonium acetate

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to fix tissues and cells and it is known as a cross-linking agent involving a wide set of amino acid residues [14, 15]. Metz et al. reported a systematic investigation on a series of synthetic peptides in the presence of glycine or $NaCNBH_3$ [16, 17]. When the reducing agent is added to the mixture after 24 h incubation with formaldehyde, besides the expected dimethylated peptide a secondary product featuring 2 mass units less can be observed whereas a Schiff-base is generated at the N-termini. According to earlier observations on acetaldehyde, this side product can be traced to an intramolecular rearrangement that leads to the formation of a *N*-methyl-4-imidazolidinone derivative, producing a mass increase of 26 Da instead of 28 [18–20].

The mild reducing agent, $NaCNBH_3$, is known to be more selective and efficient towards protonated imines and, differently from $NaBH_4$, can be used at acidic pH [21]. Jentoft and Dearborn demonstrated that the effect of pH on the reaction is more evident in case of short incubation time, with an increase in the extent of labeling in basic environments [12]. Hsu et al. were the first to report the successful application of dimethylation on peptides with a really short reaction time (5 min) and a quantitative yield using an acidic buffer (sodium acetate pH 5–6) [22]. In other papers Ji et al. adopted the same conditions, increasing the incubation time up to 1 h, while in the dimethylation after lysine guanidination approach (2MEGA), the reductive amination step is carried on at pH 8 after guanidination of lysine residues at pH 11 [23, 24]. Li et al. performed the dimethylation in water with a short reaction time, adding formaldehydes prior to $NaCNBH_3$, reporting of a side product observed when extending the incubation time [25]. In another paper they also mention negligible effects of buffer change in the range between pH 3.0 and 8.2, in a systematic investigation with formaldehyde [26]. Boersema et al. in their recent protocol for triplex experiments with formaldehydes isotopomers, recommend to work in the pH range between 5 and 8.5, while She et al. use a pH 8.5 buffer for reductive dimethylation, generating formaldehydes in situ from paraformaldehydes before the addition of the reducing agent [27, 28].

Negligible isotopic effect in HPLC runs has been verified by Hsu and afterwards by other authors anyway reporting examples concerning the elution of only a single chosen peptide. Boutilier et al. recently published a more detailed investigation confirming a minimum chromatographic resolution for the majority of eluted peptide pairs [29].

Barrios-Llerena et al. have recently published a novel labeling strategy based on diethylation employing acetaldehyde and its isotopomers bearing ^{2}H or ^{13}C. The derivatization is carried on at pH 6.6 with longer incubation time, with respect to methylation. They performed quantitation only with $^{12}C/^{13}C$ labeling that ensures negligible isotopic chromatographic effect [13]. As far as we know no earlier application of diethylation labeling have been reported except for a recent work about determination of monoamine neurotransmitters [30].

On the base of a slight variability in protocols, in terms of pH and reaction time, we decided to perform a series of tests on some commercially available peptides before undertaking experiments on complex biological samples, aiming at determine a reliable procedure. From our earlier observation with both formaldehyde and acetaldehyde we noticed that, particularly in the diethylation approach, the occurrence of side products is not unlikely at basic pH, even in the presence of a preexistent reducing environment.

2 Materials and methods

2.1 Chemicals

Water, CH_3CN, TFA, and formic acid (FA) were purchased from Romil (Cambridge, UK). Formaldehyde (37% wt. solution in H_2O) was purchased from Baker. Formaldehyde-d_2 (20% wt. solution in D_2O), acetaldehyde 99%, acetaldehyde-d_4 98%, sodium cyanoborohydride, triethylammonium bicarbonate (TEAB) solution, tetraethylammonium acetate (TEAOAc), sodium acetate (NaOAc), *o*-methyl isourea sulfate, D,L-DTT, iodoacetamide, adrenocorticothropic hormone fragment 18–39 (ACTH), and renin substrate tetradecapeptide human (TDP) were purchased from Sigma (St. Louis, MO, USA). Peptide standard mix for MALDI-TOF calibration and α-cyano-4-hydroxycinnamic acid (HCCA) were purchased from Bruker-Daltonics (Bremen, Germany). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Peptides GTFTASQNYLR, SIHVDIYSFPK, SLEVTFTPVIEDIGK, and EITFTVLASR were purchased from PSL GmbH (Heidelberg, Germany).

2.2 Peptides synthesis

Peptides **5–9** were synthesized by a fluorenylmethoxycarbonyl (Fmoc)/*tert-butyl* (*t*Bu) solid-phase peptide strategy as previously reported [31], purified to homogeneity by preparative reverse-phase HPLC, and characterized by ESI-MS-HPLC.

2.3 In solution digestion

In all steps buffers were chosen in order to avoid the introduction of other primary amino groups than those of peptides, thus no TRIS or primary ammonium salts were employed during sample treatment. 1.3 μL of 0.1 M DTT, 1.5 μL of 0.2 M iodoacetamide, and again 0.25 μL of 0.1 M DTT were subsequently added to 15 μL (100 μg) of a solution of BSA in 0.1 TEAB M, leaving the mixture for 1 h at 37°C, 1 h at room temperature in dark, and 20 min at 37°C, respectively. Samples were digested over night at 37°C by adding 2 μL of trypsin (0.5 μg/μL) quenching with 2 μL of 1% TFA.

2.4 Guanidination

Ten microliters of *o*-methyl isourea solution (0.5 M) were added to 10 μL of a peptide solution (1 mg/mL) in a

0.2 mL eppendorf tube at 60°C and pH was adjusted to 11 with 1 μL of 2 M NaOH. The reaction was kept at 60°C for 1 h and quenched with 1 μL of TFA 10%.

2.5 Reductive amination

As a general procedure for standard peptides labeling, 50 μL of reaction buffer (0,1 M) were added to 10 μL of peptide solution (1 mg/mL) in a 0.2 mL eppendorf tube. 2.5 μL of $NaCNBH_3$ (1 M) were added and the solution vortexed for 30 s, then 2.5 μL of formaldehyde solution (0.5 M) were added and the mixture vortexed again. After 1 h incubation at 25°C, the reaction was quenched with 2 μL of 10% ammonia solution to consume the excess of formaldehyde.

Ethylation was carried on with the same procedure, using 20% acetaldehyde solutions and extending the reaction time up to 6 h at 30°C. After the first 4 h incubation, a further addition of both acetaldehyde and $NaCNBH_3$ (2.5 μL each) was necessary to reach completeness.

2.6 Chromatography and MS

Before MALDI analysis all samples have been desalted by $ZipTip_{\mu\text{-}C18}$ (Millipore P10 size) using a 50% CH_3CN/0.1% TFA solution and a 0.1% TFA solution to wet and equilibrate/wash the stationary phase, respectively. Peptides were eluted on the MALDI target with a solution of HCCA (5 mg/mL in 50% CH_3CN/0,1% TFA). MALDI and LC-MALDI experiments were performed on an UltraFlex III MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics), acquiring data in positive reflectron mode. Acquisitions were performed in the mass range 200–3000 m/z with voltages of 25 and 21.7 kV for the first and second ion extraction stages, 9 kV for the lens, 26.3 and 13.8 kV for reflector 1 and 2, respectively. For CID acquisition, the instrument was switched in LIFT mode; precursor ions were manually selected for the subsequent fragmentation. MS/MS spectra were acquired with 4–8 $\times$ 10^3 laser shots using the instrument calibration file. MS and MS/MS data were processed and analyzed by the Bruker FlexAnalysis 3.0 software.(Bruker-Daltonics) operating baseline subtraction, smoothing (Savitsky–Golay) and centroiding.

LC-MALDI experiments were performed on some derivatized synthetic peptides only, with a Dionex Ultimate capillary HPLC system equipped with UV detector with U-Z View Capillary flow cell, Famos autosampler, Switchos microcolumn switching device and Probot micro fraction collector (LCPackings, Amsterdam, The Netherlands).

Ten micrograms of reaction mixture were desalted for 3 min with 0.1% TFA on a μ-precolumn cartridge C18 PepMap100 (5 mm, 300 μm id, 5 μm p.s. LC-Packing) at a flow rate of 0.030 mL/min. Gradient elution was performed on a C18 Acclaim PepMap100 (25 cm, 300 μm id, 5 μm p.s. LC-Packings) kept at 30°C. A linear gradient from 0 to 35% B in 60 min was applied at a flow rate of 3 μL/min using the following mobile phases: (i) 0.1% FA and 5% CH_3CN; (ii) 0.1% FA and 95% CH_3CN. The Probot spotting device was in-line interfaced with the UV cell output in order to automatically mix the eluate with a matrix solution (HCCA, 5 mg/mL in 50% CH_3CN/0,5% TFA) and to collect it on the MALDI target with a 30 s interval between each spot.

LC-MS experiment for quantitation were performed on a Waters nanoAquity UPLC system interfaced with a Waters Q-TOF Premier mass spectrometer equipped with a nanoESI source operating in positive mode. One microgram of BSA digest was trapped on a Symmetry C18 column (180 μm $\times$ 20 mm, 5 μm p.s., Waters) and washed for 3 min at 0.3 μL/min with 0.1% TFA. Elution was performed using a 45 min gradient with a flow rate of 250 nL/min (from 0 to 35% CH_3CN) on a BEH130 C18 nanoLC column (25 cm 75 μm id, 1.7 μm p.s., Waters). Eluent A was 0.1% FA and eluent B 0.1% FA in CH_3CN. The column temperature was set at 35°C. Lock mass ([Glu1]-fibrinopeptide B, 250 fmol/μL) was constantly infused by the nanoAcquity auxiliary pump at a flow rate of 250 nL/min. The mass spectrometer was set to perform automated data-dependent acquisition selecting five precursors for every MS survey scan. Source capillary voltage was set to 2.6 kV, temperature: 90°C, cone gas: 25 L/h, nano flow gas: 0.62 bar. Analyzer was set to operate in the V mode. Survey scans were acquired in the mass range 200–1800 m/z with a scan time of 0.8 s and an interscan delay of 0.1 s. MS/MS scans were acquired in the mass range 200–2200 m/z with the same scan rate as the survey scans. A dynamic exclusion window was set to 30 s.

2.7 Data bank search and quantitation

LC-MS/MS data were processed using the Mascot Distiller package; default processing options were applied. Databank searches were performed within the SwissProt database (released version: 2012_09) selecting "other mammalia" as taxonomic restriction. Precursor tolerance of 20 ppm and fragment tolerance of 0.1 Da were set. Cysteine carbamidomethylation and methionine oxidation were set as fixed and variable modification, respectively; according to the sample the specific modifications induced by the labeling strategy were considered as variable. Dimethyl (K), dimethyl (N-term), dimethyl:2H(4)(K), dimethyl:2H(4) (N-term), diethyl (K), diethyl (N-term), diethyl:2H(8) (K); diethyl:2H(8) (N-term) were selected among the Mascot Server panel. Four supplemental modifications regarding the imidazolidinone derivatives observed in the methylation and ethylation reactions were edited using the configuration editor of the Mascot Server. Relative quantitative analysis were carried out measuring the ratio between the signals of light and heavy labeled peptides (L/H) in the mass spectra, using the Quantitation Tool of Mascot Distiller. Di-methylation and diethylation were pre-existing quantitation methods while two further methods were edited to perform the quantitation of the imidazolidinone derivative in methylation reactions.

3 Results and discussion

Early tests have been performed with nondeuterated reagents only, on four synthetic proteotypic peptides from mouse plasma proteins, and two commercially available nontryptic peptides: GTFTASQNYLR (**1**, exact mass [EM]: 1256.6), EITFTVLASR (**2**, EM: 1135.6), SIHVDIYSFPK (**3**, EM: 1304.7), SLEVTFTPVIEDIGK (**4**, EM: 1646.9), DRVYIHPFHLVIHN (renin substrate tetradecapeptide human, **TDP**, EM: 1758.9), and RPVKVYPNGAEDESAEAFPLEF (adrenocorticothropic hormone fragment 18–39, **ACTH**, EM: 2464.2). Peptides **1**, **2**, and **TDP** present a single reactive site at the N-terminus, while peptides **3** and **4** contain a C-terminal lysine that provides a further derivatizable amino group, and **ACTH** features an internal lysine together with an arginine and a tyrosine, both close enough to be possibly involved in intramolecular cross-links [19]. All the final reaction mixtures were analyzed by MALDI-TOF-MS to verify the presence of the desired product and the complete absence of the starting material.

3.1 Preliminary observations

In a preliminary experiment dimethylation was performed on **TDP** and **ACTH** by adding formaldehyde prior to $NaCNBH_3$ and using 0.1 M TEAB (pH 8.8) as reaction buffer. With **TDP** we observed two partially overlapped signals in the MALDI spectrum, corresponding to the expected dimethylated product ($[M+H]^+$: 1787.9 m/z), with a mass increase of 28 Da, and to a much less abundant side product featuring 2 mass units less (Supporting Information Fig. 1). A 26 Da mass increment is consistent with a rearrangement involving the Schiff-base to generate an imidazolidinone derivative at the N-terminus with the formation of a methylene bond that implies the loss of two hydrogens [22] (Fig. 1). In the same conditions the signal of a secondary product with a 52 Da mass increase is observed with **ACTH**, besides the signal of the expected tetra-methylated peptide ($\Delta m = +56$ Da, $[M+H]^+$: 2521.3 m/z; Supporting Information Fig. 2). In this case the apparent loss of four hydrogens can be ascribed to the formation of two methylene bridges in an intramolecular cross-link that probably involves the Lys^4 and the N-terminal arginine [16] (Fig. 2). Interestingly, the adduct formation is almost completely suppressed when formaldehyde is added after $NaCNBH_3$, namely when the imine is formed in a preexistent reducing environment. No side products have been observed performing the derivatization in acidic conditions, using a 0.1 M NaOAc buffer (pH 5.3), whereas a small +26 Da signal is still visible in the MALDI spectrum of **TDP** at neutral pH (6.9) with 0.1 M TEAOAc.

Derivatization of **TDP** and **ACTH** with acetaldehyde has been set up modifying the protocol adopted by Boersema et al. with formaldehyde. Reaction time is noticeably longer with respect to methylation, due to the reduced electrophilicity of the Shiff-base, and completeness is achieved, at pH 5.3, after 6 h incubation at 30°C. In this case, higher pH values

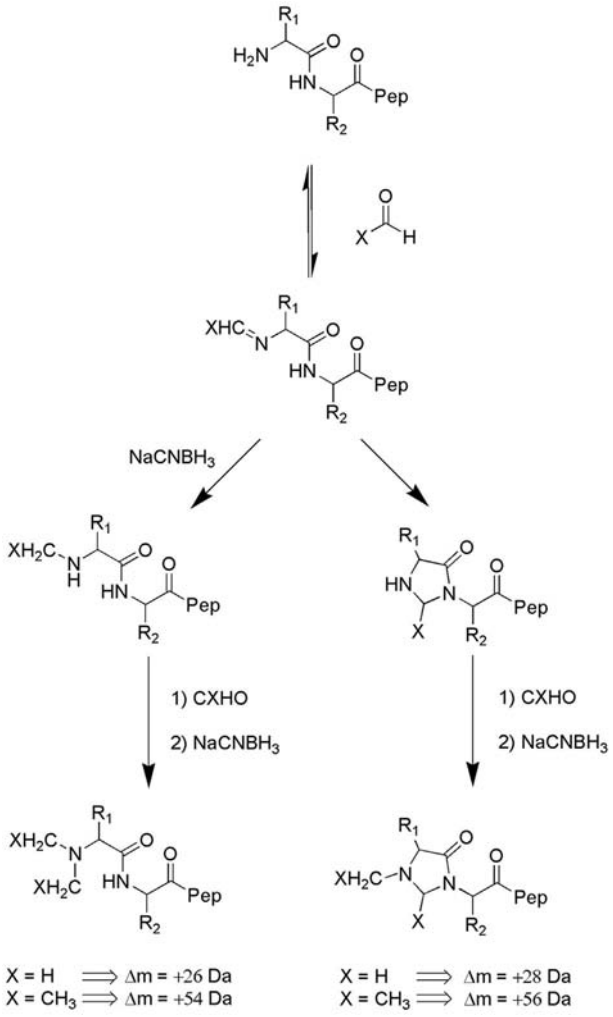

Figure 1. The *N*-alkyl-4-imidazolidinone derivative can be generated by the nucleophilic addition of the contiguous peptidic nitrogen to the Shiff-base before it can be reduced by hydride.

produce either the formation of a side product and a further slowdown of the reaction. Indeed a +54 Da product becomes evident with **TDP**, partially overlapped with the expected +56 Da diethylated compound ($[M+H]^+$: 1815.9 m/z), becoming even the most abundant observed when 0.1 M TEAB (pH 8.5) is employed as reaction buffer (Supporting Information Fig. 1). In any condition no secondary product is detected with **ACTH** beside the expected tetra-ethylated compound ($[M+H]^+$: 2576.3 m/z). In this case the imidazolidinone rearrangement is forbidden while the intramolecular cross-link is probably prevented by steric reasons (Supporting Information Fig. 2).

3.2 Investigation on side products nature

The peculiar modification observed on **ACTH** with formaldehyde was studied more in detail by means of five ad hoc synthesized decapeptides designed to reproduce its presumed

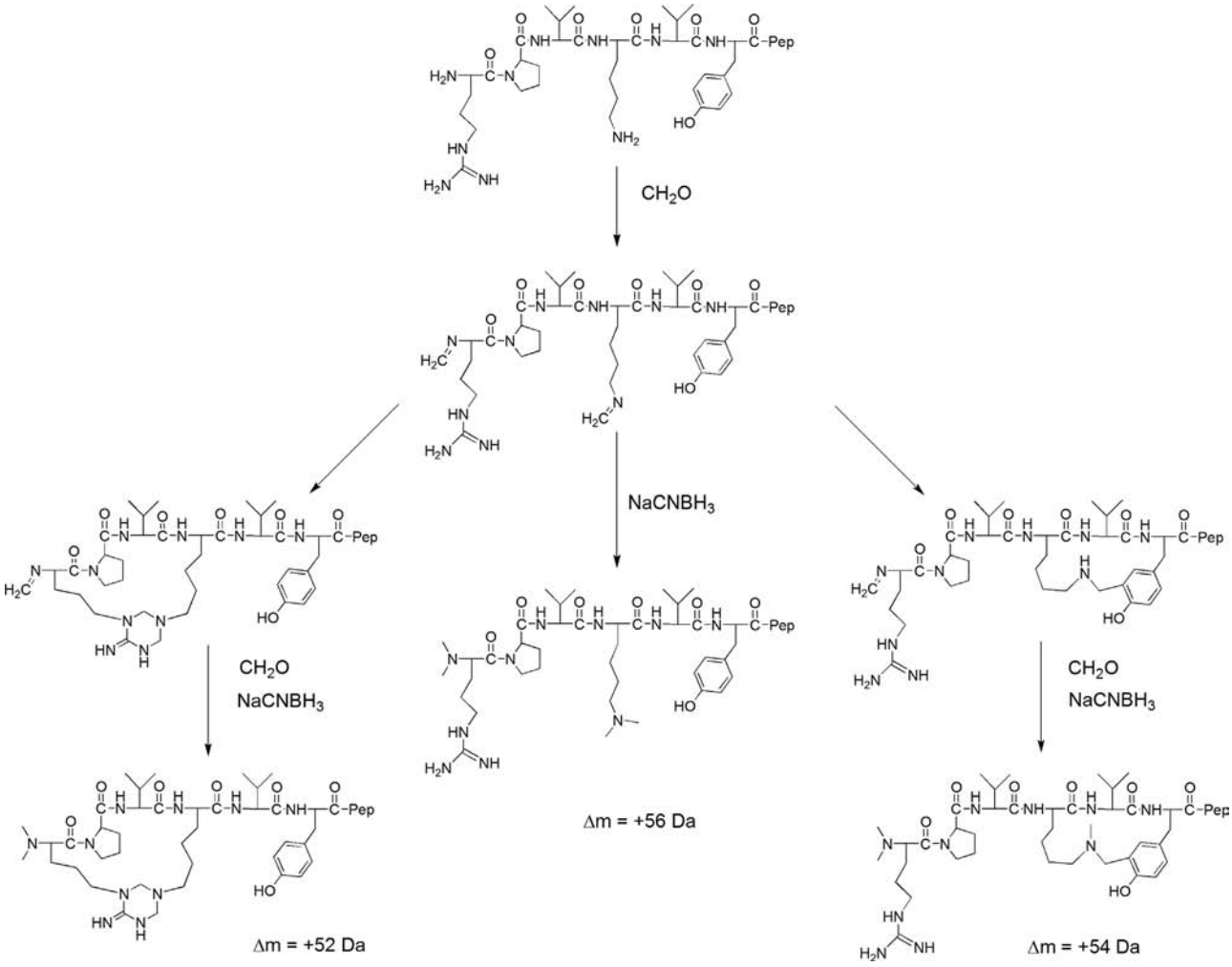

Figure 2. Possible reaction paths for **ACTH** in the reaction with formaldehyde. Intramolecular cross-link reactions can take place if $NaCNBH_3$ is added after a while.

reactive region. RPVKVYPNGA (**5**, EM: 1099.6) was prepared to replicate the same sequence of **ACTH** from the N-terminus to the Ala^{10}, while its N-protected homologous, AcRPVKVYPNGA (**6**, EM: 1141.6), was thought to restrict the derivatization to Lys^4. With APVKVYPNGA (**7**, EM: 1014.6) and AcAPVKVYPNGA (**8**, EM: 1056.6) we aimed at preventing the intramolecular cross-link by replacing Arg^1 with Ala, while AcAPVKVAPNGA (**9**, EM: 964.5) features the replacement of Tyr^6 with Ala, eliminating a further possible residue that could be involved in a reaction with the dimethylated Lys^4 (Fig. 2).

A mixture of peptides **1–5** was submitted to reductive amination either with formaldehyde and acetaldehyde changing four different buffers: NaOAc (0.1 M, pH 5.3), TEAOAc (0.1 M, pH 6.9), TEAB (0.1 M, pH 8.0), and TEAB (0.1 M, pH 8.8). In MALDI spectra we observed a general trend in accordance with our early experiments, detecting the presence of a side product increasing pH, again with a much higher easiness with acetaldehyde than with formaldehyde. Signals of the +26 or +54 Da products in the reaction with formaldehyde become perceptible at basic pH and, from a rough estimation, are always below 10% intensity with respect to the desired di- or tetra-methylated products. In the ethylation approach these signals are instead clearly visible yet at pH 7.0 and can become almost exclusive above pH 8.0. In the case of **5**, long incubation time in a basic environment seems also to give partial deamidation of Asn^8 (Fig. 3). It is clear that the chemical environment of the primary amino groups plays a crucial role in regulating the competition between the intermolecular addition of hydride and other intramolecular or intermolecular nucleophilic attacks to the generated imine. Peptide **1** yields quite only the expected product ($[M+H]^+$ 1285.6 m/z) in the reaction with formaldehyde and $NaCNBH_3$ even at basic pH, while in the reaction with acetaldehyde the signal intensity of the side product ($[M+H]^+$ 1311.7 m/z) is about 5% at pH 8.0 and slightly increases at pH 8.8. On the other hand, a small amount of secondary product is visible when formaldehyde reacts with peptides **2**, **3**, and **4** at pH 8.0

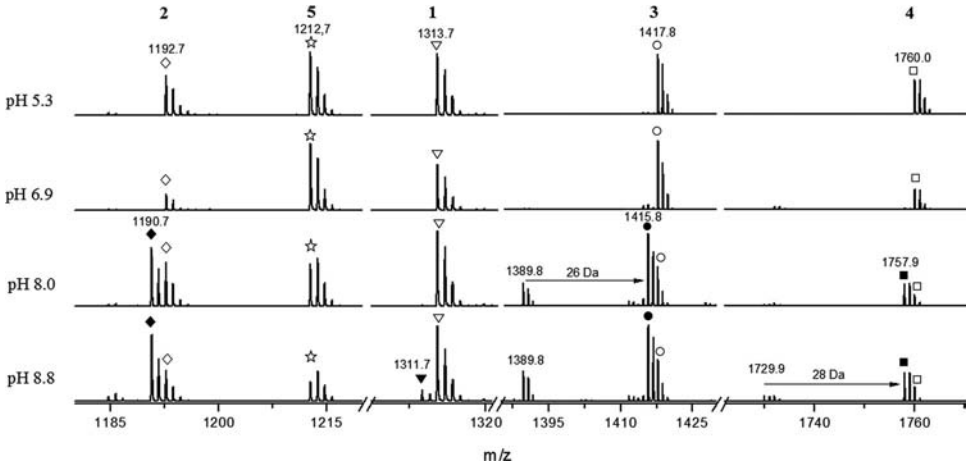

Figure 3. MALDI spectra showing different behavior of peptides **1–5** in the reductive amination with acetaldehyde. The expected di- or tetra-ethylated products are tagged with empty symbols. Side products signals (color filled symbols) increase in intensity and can even become the main ones while reactions slow down.

but it becomes considerable with acetaldehyde. Such products are the main ones at pH 8.8, where completeness is not always achieved even after 6 h incubation (see Fig. 3).

To get a first indication about the side-reaction location the 2MEGA approach was applied on peptides bearing a lysine. If a cyclization at the N-terminus occurred, it should be observed as well when lysine is guanidinated before the dimethylation step at pH 8.0, and indeed a +26 Da signal is still detectable in the MALDI mass spectra of peptides **3** and **4**.

Both dimethylation and diethylation were performed on peptides **5–9** adding the aldehydes before $NaCNBH_3$ in order to let the possible cyclization between Arg^1 and Lys^4 be favored, and the side product be more perceptible in the mass spectra (Supporting Information Fig. 3). In the reaction with acetaldehyde, we never observed the formation of a cross-link product even if the reducing agent was added 3 h after the introduction of acetaldehyde at pH 8.5. Steric hindrance is probably the main reason for this difference with dimethylation, together with an apparent overall reduced reactivity.

Peptide **5** behaves exactly as **ACTH** in the reaction with formaldehyde, thus a couple of products (+56 Da: $[M+H]^+$ 1156.6 m/z, +52 Da: $[M+H]^+$ 1154.6 m/z) is observed only above pH 8.0. Similarly, **6** yields the expected dimethylated product ($[M+H]^+$ 1170.6 m/z) together with the respective side product with 4 mass units less. A single tetra-methylated product is observed with **7** ($[M+H]^+$ m/z), while **8** and **9** give a single dimethylated derivative at the lysine residue ($[M+H]^+$ 1085.6 m/z and $[M+H]^+$ 993.6 m/z, respectively). All the mass shift observed in the dimethylation, diethylation, and 2MEGA approaches with peptides **1–9** are summarized in Table 1.

It was very surprising to detect the occasional appearance of a +26 Da signal in the MALDI spectra of peptides **5–9** after reaction with formaldehyde either at basic and acidic pH. (Supporting Information Fig. 4) As no evidence of a corresponding product is found in the ESI-MS spectra, this phenomenon seems to be related to a rearrangement that could take place directly in the MALDI source (Fig. 4). Laser desorption is known to be "harder" than ESI, so what we observed was probably the loss of two hydrogens by the produced tertiary amine to generate an immonium ion that gives a $[M-H]^+$ signal [32] (Supporting Information Fig. 5). Slight differences have been observed by changing the MALDI matrix, but anyway we did not succeed in controlling the reaction probably because it is not driven by a single factor (data not shown). This immonium ion rearrangement seems anyway to be a really unpredictable and unreproducible

Table 1. Mass increments (amu) observed in the MALDI spectra of reaction products at two different pH values

Peptide \ Reaction	Dimethylation		2MEGA		Diethylation	
	pH 5.5	pH 8.5	pH 5.5	pH 8.5	pH 5.5	pH 8.5
GTFTASQNYLR (**1**)	28	28, 26	—	—	56	56, 54
EITFTVLASR (**2**)	28	28, 26	—	—	56	56, 54
SIHVDIYSFP**K** (**3**)	56	56, 54	70	70, 68	112	112, 110
SLEVTFTPVIEDIG**K** (**4**)	56	56, 54	70	70, 68	112	112, 110
RPV**K**VYPNGA (**5**)	56	56, 52[a)]	70	70	112	112
AcRPV**K**VYPNGA (**6**)	28	28, 24[a)]	—	—	56	56
APV**K**VYPNGA (**7**)	56	56	70	70	112	112
AcAPV**K**VYPNGA (**8**)	28	28	—	—	56	56
AcAPV**K**VAPNGA (**9**)	28	28	—	—	56	56

a) Observed only if formaldehyde is added before $NaCNBH_3$.

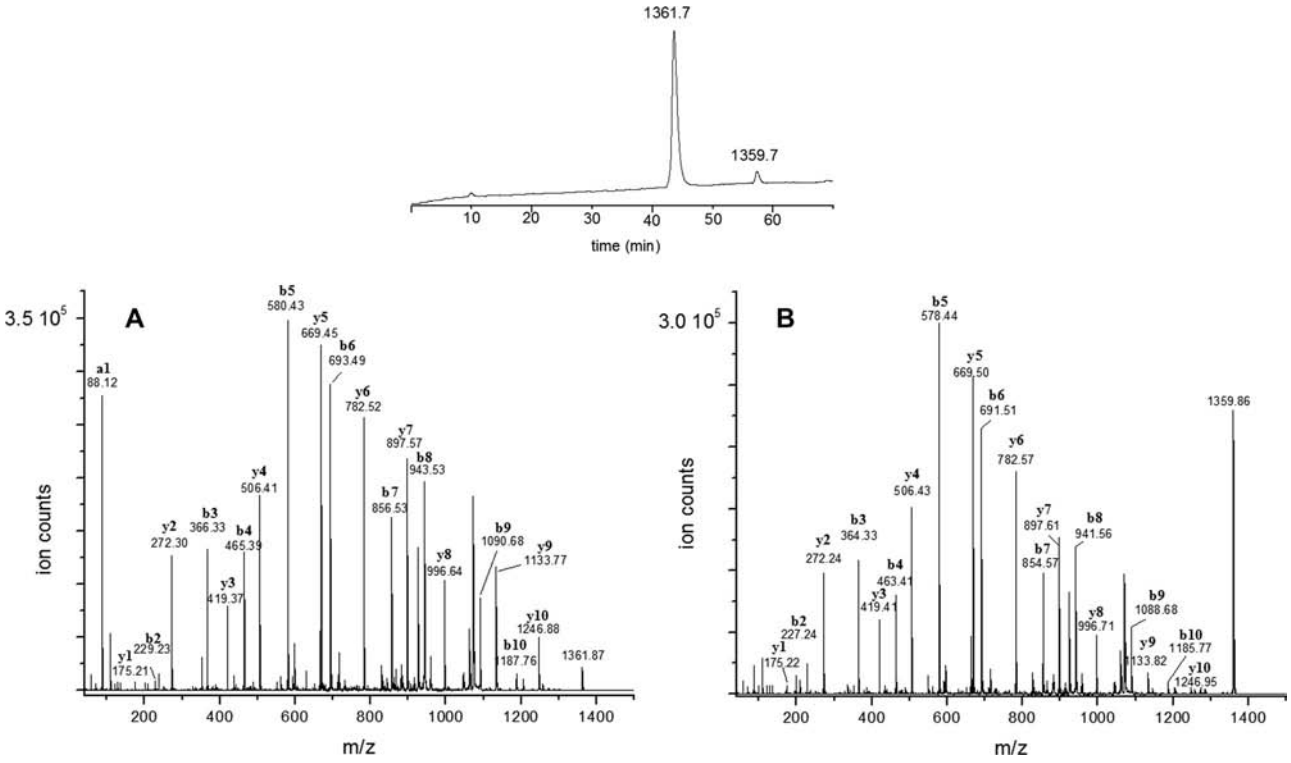

Figure 4. Chromatogram and MALDI-CID spectra of the two products of reductive dimethylation of **3**. (A) First eluted compound. (B) Second eluted compound.

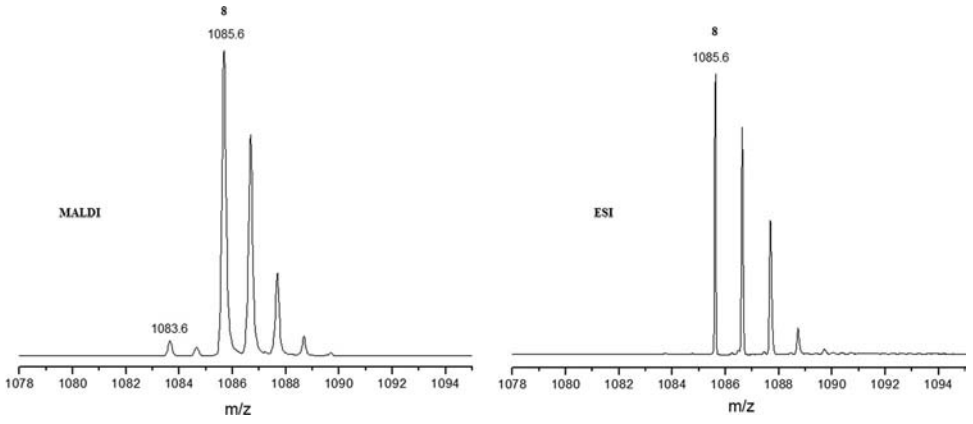

Figure 5. The $[M-H]^+$ signal observed in the MALDI-TOF spectrum of dimethylated **8** (left) is not present in the ESI-QTOF spectrum of the same reaction mixture (right).

phenomenon that generates a small amount of subproduct and could be considered a minor hitch in quantitative analysis by MALDI experiments only. LC-MALDI experiments were performed with a few reaction mixture of single synthetic peptides, where a side product was detected, aiming at verifying if the apparent $[M-H]^+$ signal could be referred to the imidazolidinone derivative or to the "in source" generated immonium ion. The CID spectra of the two-reaction product of **3**, give a further indication about the location of the rearrangement. In the MS/MS spectrum of the first eluted product (tetramethylated **3**, $[M+H]^+$ 1361.7 m/z) the expected enhancement of the a_1 ion is observed [33], the *y* series is completely detected, and in the *b* series the b_1 ion misses. The *y* sequence is exactly the same in the spectrum of the second eluted compound ($[M+H]^+$ 1359.7 m/z), whereas all the *b* ions are shifted by 2 mass units (Fig. 5).

3.3 Quantitation

Although we outlined here how reaction conditions can be optimized to reduce or completely suppress such unwanted products on standard peptides, an initial evaluation of their impact in quantitation experiments has been attempted with a BSA digest. Differential labeling was performed with formaldehyde at both acidic and basic pH, mixing the two final solutions in a 5:2 ratio. Quantitative analysis by LC-MS/MS experiments yielded in both cases L/H values

Table 2. Mascot Distiller quantitation reports for differential BSA labeling with formaldehyde at two different pH values

BSA	pH 5.5					pH 8.5				
	Score 1291	L/H_T[a] 2.5	L/H_E[b] 2.52		SDg[c] 1.12	Score 1051	L/H_T[a] 2.5	L/H_E[b] 2.47		SDg[c] 1.13
Peptide sequence	L/H	SD	*z*	*m/z*	Score	L/H	SD	*z*	*m/z*	Score
CCAADDKEACFAVEGPK	2.6	0.04	3	675.32	54.9					
CCTESLVNR	2.6	0.01	2	583.75	47.6					
DAFLGSFLYEYSR	2.5	0.01	2	798.37	74.7	2.3	0.02	2	798.39	62.2
DDPHACYSTVFDK	2.7	0.07	3	537.58	39.9					
DDSPDLPK	2.5	0.05	2	471.74	26.3					
DLGEEHFK						2.3	0.05	3	344.17	14.9
DLGEEHFK						2.4	0.06	2	515.76	31.3
ECCHGDLLECADDR	2.5	0.03	3	593.23	76.6	2.5	0.01	3	593.23	76.5
ECCHGDLLECADDRADLAK						2.9	0.03	3	768.66	55.5
EYEATLEECCAK	2.8	0.03	2	779.84	68.1	2.9	0.03	2	779.83	68.9
GLVLIAFSQYLQQCPFDEHVK	2.9	0.07	3	850.12	48.6	2.2	0.01	3	850.10	78.8
KQTALVELLK						2.6	0.03	2	613.90	26.7
KVPQVSTPTLVEVSR						2.9	0.04	2	565.99	56.1
LCVLHEK						2.1	0.02	2	481.79	44.2
LFTFHADICTLPDTEK	2.2	0.06	3	655.32	43.5	2.3	0.03	3	655.33	33.5
LGEYGFQNALIVR						2.5	0.03	2	754.41	44.8
MPCTEDYLSLILNR	2.6	0.04	3	584.96	42.6	2.5	0.06	3	584.95	39.8
MPCTEDYLSLILNR	2.8	0.04	2	876.93	70.0	2.7	0.06	2	876.93	54.1
QNCDQFEK	2.6	0.03	2	562.75	54.9	1.9	0.04	2	566.77	45.1
QTALVELLK	1.8	0.03	2	539.86	62.4	2.2	0.01	2	539.86	54.2
RHPYFYAPELLYYANK	2.6	0.05	3	701.03	38.2	2.9	0.06	3	701.02	38.0
SLHTLFGDELCK	2.5	0.05	2	738.38	71.8					
TCVADESHAGCEK	2.5	0.01	2	760.33	68.7					
TVMENFVAFVDK	2.5	0.03	2	728.37	71.1	2.0	0.07	2	732.40	52.7
YICDNQDTISSK	2.2	0.02	2	754.37	104.2	2.7	0.01	2	750.35	75.6

Identified peptide pairs with evident outlier L/H values or with SD $>$ 0.1 were excluded from the analysis.
a) Theoretical L/H.
b) Experimental L/H.
c) Geometric standard deviation.

in great accordance with the theoretical values and similar protein sequence coverage (Table 2). No evidences of side-products effects rose from these results, and indeed very few peptides were detected when the imidazolidinone rearrangement was set as fixed modification in the Mascot search. Concerning the reaction carried on at pH 8.5, according with the standard error threshold that we set to perform quantitation (SD $\leq$ 0.1), only two peptide pairs were suitable to the analysis; however, yielding a L/H slightly below the theoretic value (Supporting Information Table 1). Performing the diethylation labeling on BSA digest at pH 5.5, 7.0, and 8.5 we could not achieve reliable quantitation results, as in any case the analysis yields L/H values varying a lot from one peptide to another and being often largely above the expected value. Nevertheless, we definitely identified several peptides bearing the imidazolidinone rearrangement yet at neutral conditions, frequently associated to a high score.

4 Concluding remarks

With our work we aimed at rationalizing the various ensemble of published procedures for reductive amination, pointing out the issue of side reactions on the base of some observations on standard peptides. We showed that the undesired occurrence of side products is easier than as reported when pH is maintained above 7.0, either with formaldehyde and much more manifestly with acetaldehyde. We anyway demonstrated that working with formaldehyde, in a pH range that includes all the conditions reported in the literature (pH 5–8.5), should not bring to any significant drawback when attempting quantitation on a more complex sample. On the other hand, we are also able to state that diethylation brings to large amounts of side product featuring N-terminal cyclization, when performed at basic pH, but also at neutral conditions. Although the real impact on quantitation must be still evaluated with experiments on more complex samples, our work has

disclosed some crucial aspects of the labeling procedure that have never been taken into consideration before.

By a chemical point of view the observations on **ACTH** or its mimicking peptides **5–9** toward formaldehyde are undoubtedly peculiar and interesting but, fortunately, such rearrangements should be very uncommon when a tryptic digest is subjected to reductive amination. An intramolecular cross-link between arginine and lysine can take place only if a miscleavage occurs or if Lys-C is used for digestion. Moreover, both residues must be localized at a proper distance each other in the peptide sequence.

Our study has partially highlighted how complex the scenario can be when we undertake a derivatization task on a complex ensemble of substrates. In our experiments, we have shown that reductive amination on tryptic peptides leads to the desired dimethylation, or diethylations, at N-termini and at lysine residues only when pH ensures a rapid action of the hydride and reduces the nucleophilicity of other amino groups. With these initial evidences, based on our controlled model experiments, we cannot describe a comprehensive proteome picture, or make an assessment of the residues that are more likely to promote a rearrangement at the N-terminus, or even spot-out the lysine residues more likely involved in cross-links. A further more extensive and systematic investigation could be interesting to widen the knowledge on aldehyde-induced modification on peptides and proteins.

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