Systematic Evaluation of Protein Reduction and Alkylation Reveals Massive Unspecific Side Effects by Iodine-containing Reagents*

Torsten Müller‡§, and Dominic Winter‡¶

Reduction and alkylation of cysteine residues is part of virtually any proteomics workflow. Despite its frequent use, up to date no systematic investigation of the impact of different conditions on the outcome of proteomics studies has been performed. In this study, we compared common reduction reagents (dithiothreitol, tris-(2-carboxyethyl)-phosphine, and β-mercaptoethanol) and alkylation reagents (iodoacetamide, iodoacetic acid, acrylamide, and chloroacetamide). Using in-gel digests as well as SAX fractionated in-solution digests of cytosolic fractions of HeLa cells, we evaluated 13 different reduction and alkylation conditions resulting in considerably varying identification rates. We observed strong differences in offsite alkylation reactions at 7 amino acids as well as at the peptide N terminus, identifying single and double adducts of all reagents. Using dimethyl labeling, mass tolerant searches, and synthetic peptide experiments, we identified alkylation of methionine residues by iodine-containing alkylation reagents as one of the major factors for the differences. We observed differences of more than 9-fold in numbers of identified methionine-containing peptide spectral matches for in-gel digested samples between iodine- and noniodine-containing alkylation reagents. This was because of formation of carbamidomethylated and carboxymethylated methionine side chains and a resulting prominent neutral loss during ESI ionization or in MS/MS fragmentation, strongly decreasing identification rates of methionine-containing peptides. We achieved best results with acrylamide as alkylation reagent, whereas the highest numbers of peptide spectral matches were obtained when reducing with dithiothreitol and β-mercaptoethanol for the in-solution and the in-gel digested samples, respectively. Molecular & Cellular Proteomics 16: 10.1074/mcp.M116.064048, 1173–1187, 2017.

Mass spectrometry (MS) has become one of the major techniques for the qualitative and quantitative analysis of proteins. Because the introduction of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI)1 (1, 2), which paved the way for modern mass spectrometry-based proteomics, the power of mass spectrometric analyses has improved continuously. The performance of chromatography systems and mass spectrometers, as well as the development of novel sample preparation protocols are the main contributing reasons. Bottom-up proteomics is the most common method for protein identification and characterization by mass spectrometry. It is based on the enzymatic digestion of proteins followed by the subsequent analysis of proteolytic peptides.

In current bottom-up proteomics strategies, all sample preparation protocols have several key elements in common: (1) cells or tissues are lysed and proteins are extracted; (2) proteins are reduced in order to break disulfide bonds; (3) proteins are alkylated to covalently modify cysteine SH-groups, preventing them from forming unwanted novel disulfide bonds; and (4) proteins are enzymatically digested to peptides. Dependent on the complexity of the sample and the goal of the analysis, samples are frequently fractionated on the protein- or the peptide-level. One common approach is the separation of proteins according to their mass using SDS-PAGE followed by in-gel digestion (3). Alternatively, proteins are digested in-solution followed by the fractionation of peptides using isolectric focusing or chromatography-based approaches (4). If specific modifications are to be analyzed, enrichment methods are commonly used; for example, phosphorylated peptides are enriched using antibodies or metal-affinity resins (5).

Cell lysis, protein purification, proteolytic digestion, and peptide fractionation have been optimized in several studies (e.g., 4, 6–8). The reduction and alkylation step of proteins, however, has surprisingly not been systematically investigated up to date, despite its use in virtually any proteomic

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1 The abbreviations used are: MALDI, matrix-assisted laser desorption ionization; IAA, iodoacetamide; IAC, iodoacetic acid; AA, acrylamide; CAA, chloroacetamide; DTT, dithiothreitol; TCEP, tris-(2-carboxyethyl)-phosphine; BME, β-mercaptoethanol; NEM, N-ethylmaleimide; MMTS, methyl methanethiosulfonate; PSM, peptide spectrum matches; FASP, filter aided sample preparation; can, acetonitrile; FA, formic acid; SAX, strong anion exchange; TEAB, triethylammoniumbicarbonate.
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experiment (excluding experiments investigating disulfide bridges or cysteine modifications, for example (9)).

The most common reducing reagent used in proteomics experiments, dithiothreitol (DTT), was introduced in 1964, as a more stable and less toxic alternative to commonly used reducing reagents such as glutathione or β-mercaptoethanol (10). The second most common reducing reagent used by the community, tris-2-(carboxyethyl)-phosphine (TCEP), was introduced in 1999 (11). In this study, the authors showed that TCEP is less susceptible to oxidation than DTT and superior in conserving enzymatic activity using myosin as an example. Finally, β-mercaptoethanol (BME) is frequently used as reducing reagent among molecular biologists, for example, as part of the standard buffer for SDS-PAGE sample preparation (Laemmli buffer (12)), whereas it is rarely used in sample preparation for mass spectrometry-based proteomics.

After reduction of disulfide bridges, alkylation of the free SH-groups is usually performed by iodoacetamide (IAA, (3)). Because of a variety of side reactions that have been observed for IAA (13) several structurally related alternatives have been introduced, including iodoacetic acid (IAc), chloroacetamide (CAA), and acrylamide (AA), as well as the structurally not related reagents N-ethylmaleimide (NEM), methyl methanethiosulfonate (MMTS), and 4-vinylpyridine (14–16). Early studies performed with standard protein digests and MALDI mass spectrometry did not identify differences concerning the performance of the different alkylation reagents (14), whereas later studies identified several side products of the carbamylation reaction using IAA (13, 17–19). Based on these studies, one would expect that the above-mentioned alternative alkylation reagents (IAc, AA, and CAA) should be used frequently to minimize the number of chemical artifacts. The authors have the impression, however, that the combination of DTT and IAA is nevertheless the most frequently used combination in the field of mass spectrometry-based proteomics (supplemental Fig. S1 and supplemental Table S1).

In this study, we systematically evaluated reduction and alkylation of proteins using three reducing agents (DTT, TCEP, and BME) in combination with four alkylation agents (IAA, IAC, AA, and CAA). We tested these conditions using strong anion exchange (SAX) fractionated in-solution digests and in-gel digested fractions of samples enriched for cytosolic proteins of HeLa cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture/Preparation of Cytosolic Fraction**—HeLa cells were grown on 15 cm cell culture dishes in DMEM, supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, until confluency. Cells were washed 3 times with ice cold PBS, harvested by scraping in 1 ml ice cold sucrose buffer (250 mM sucrose, 15 mM KCl, 1.5 mM MgAc, 10 mM HEPES, 1× Complete, Roche Diagnostics, Berlin, Germany), and homogenized using a dounce homogenizer on ice (30 strokes). The solution was centrifuged at 1000 × g, 4 °C for 10 min, and the supernatant was transferred to a new 1.5 ml tube, whereas the post nuclear pellet was re-suspended in 1 ml sucrose buffer for a second round of homogenization and centrifugation. The supernatants from both homogenization steps were combined and centrifuged at 10,000 × g, 4 °C for 10 min. The supernatant was transferred to a new tube and another centrifugation step centred at 100,000 × g, 4 °C for 1 h was performed. The supernatant (cytosolic fraction) was mixed with acetone (–20 °C) in a ratio of 1 to 4 (v/v), vortexed, and incubated at –20 °C overnight. The next day, samples were centrifuged at 20,000 × g, 4 °C for 30 min, the supernatant discarded, and the pellet air dried at 23 °C. The pellet was re-solubilized in 0.1 M TRIS-HCl/4% SDS by heating at 95 °C for 5 min, centrifuged at 20,000 × g, 23 °C for 30 min, and the clear supernatant transferred to a new tube. The protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, CA). We generated enough cytosolic fraction in one batch to perform all experiments. The resulting sample was partitioned in several aliquots and stored at –20 °C until they were used for the individual experiments.

**In-solution Reduction, Alkylation, and Digestion**—For each experimental condition, 100 μg of protein were used. Protein reduction was performed for 30 min at 56 °C with 5 mM DTT, 5 mM TCEP, or 10 mM BME, respectively. Subsequent alkylation was performed in the dark for 30 min at 23 °C with 20 mM IAA, IAC, AA, or CAA, respectively. Samples were quenched by addition of DTT, TCEP, or BME, and digested in-solution by filter aided sample preparation (FASP), using 30 kDa molecular weight cutoff filters (Microcon, Merck Millipore, Billerica, MA) as described elsewhere (20). Briefly, samples were mixed with 200 μl of 8 M urea in TRIS-HCl, pH 8.5, loaded in the filtration devices, and centrifuged at 14,000 × g for 15 min. Four additional centrifugation steps were performed using buffer containing 8 M urea followed by 3 steps with 100 μl 0.05 M NH4HCO3 pH 7.8. Finally, 1 μg trypsin (Promega, Madison, WI) was added to each sample in 60 μl 0.05 M NH4HCO3 and incubated in a wet chamber overnight at 37 °C (enzyme/protein ratio 1:100). Peptides were eluted from the filtration devices and desalted using Oasis HLB 1 cc 10-mg cartridges (Waters, Milford, MA) as described elsewhere (8). Briefly, anion exchange microcolumns were manufactured by stacking 12 layers of 3 mm Empore anion exchange disks (3M, Maplewood, MN) into a 200 μl micropipette tip. Microcolumns were equilibrated by consecutive centrifugation steps at 5000 × g for 3 min using 100 μl MeOH, 100 μl 1 mM NaOH, and 100 μl Britton & Robinson buffer (BR buffer; 20 mM acetic acid, 20 mM phosphoric acid, and 20 mM boric acid) adjusted to pH 11. Peptides were dissolved in 100 μl BR buffer, pH 11, loaded on the microcolumns, and eluted stepwise with 100 μl of BR buffer adjusted to pH 8, 6, 5, 4, and 3. Fractions were collected on Stage tips (21) containing 6 layers of 3 mm Empore C18 membrane. Stage tips were washed with 50 μl 0.1% trifluoroacetic acid (TFA) and peptides eluted with 40 μl 60% acetonitrile (ACN), dried in a vacuum centrifuge, and resuspended in 20 μl 5% ACN, 5% formic acid (FA).

**In-gel Reduction, Alkylation, and Digestion**—For each condition 50 μg of protein, mixed in a 1:4 (v/v) ratio with sample loading buffer (0.25 M TRIS-HCl pH 6.8, 8% SDS, 40% Glycerol, and 0.004% Bromphenol blue), was loaded on a 10% SDS-PAGE gel. Electrophoresis was performed, the gels washed 2 times with ddH2O, and stained using Coomassie brilliant blue (Page Blue Protein Staining Solution, Thermo Fisher Scientific, Waltham, MA) overnight. Gels were destained using ddH2O, the same gel section excised for each sample, cut into cubes of ~1 mm3, and transferred to 1.5 ml micro tubes. Gel pieces were destained twice using 1 ml of 30% ACN, 0.07 M NH4HCO3 at 25 °C, 800 rpm for 30 min, and reduced using 100 μl of either 20 mM DTT, 20 mM TCEP, or 40 mM BME in 0.1× NH4HCO3 at 56 °C for 45 min. After removal of the reducing reagent, 100 μl of...
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55 mM IAA, IAC, AA, or CAA in 0.1 M NH₄HCO₃ were added and samples incubated at 23 °C for 30 min in the dark followed by washing with 0.1 M NH₄HCO₃ at 800 rpm, 23 °C for 15 min. Subsequently, gel pieces were dehydrated by ACN and dried using a vacuum centrifuge. To each sample, 1 μg of trypsin (Promega) in 0.1 M NH₄HCO₃ was added and incubated overnight at 37 °C. Peptides were extracted from the gel pieces using an optimized protocol (6). Briefly, gel pieces were incubated consecutively with (1) 50 μl 0.1% TFA, 50% ACN, (2) 50 μl 0.1 M NH₄HCO₃, and (3) 100 μl 100% ACN at 25 °C, 800 rpm for 15 min. Supernatants were combined, dried using a vacuum centrifuge, resuspended in 100 μl 0.01% acetic acid, 3% ACN, and desalted using C₁₈ stage tips (21). Peptides were eluted twice using 20 μl 0.5% acetic acid, 80% ACN, eluate fractions dried using a vacuum centrifuge, and resuspended in 20 μl 5% ACN, 5% FA.

LC-MS/MS Analyses—Analyses were performed using an EASY-nLC 1000 UHPLC system in combination with an Orbitrap Velos mass spectrometer (both Thermo Fisher Scientific). Five-microliter samples were loaded directly on in-house manufactured analytical columns (spray tips generated from 360 μm outer diameter and 100 μm inner diameter fused silica capillaries using a P-2000 laser puller (Sutter Instruments, Novato, CA) packed for 20 cm with 5 μm Reprosil AQ C₁₈ particles (Dr. Maisch, Ammerbuch, Germany) at a flow rate of 1 μl/min using 100% solvent A (0.1% FA in water). Peptides were eluted using linear gradients from 99% solvent A, 1% solvent B (ACN with 0.1% FA) to 65% solvent A, 35% solvent B in 30 min for the SAX fractions and 60 min for the in-gel digests. Eluting peptides were ionized in the positive ion mode at 1.6 kV and MS survey scans from m/z 400 to m/z 1200 acquired at a resolution of 30,000 in the Orbitrap analyzer. CID-MS/MS spectra of the top 10 most abundant ions were performed in the ion trap and precursor ions excluded from further fragmentation for 30 s. For MS/MS scans, neutral losses of 105 (IAA and IAC), and 119 (AA) were defined.

Fragmentation for 30 s. For MSA scans, neutral losses of 105 (IAA and IAC), and 119 (AA) were defined. CID-MS/MS spectra of the top 10 most abundant ions were performed in the ion trap and precursor ions excluded from further fragmentation for 30 s. For MS/MS scans, neutral losses of 105 (IAA and IAC), and 119 (AA) were defined. CID-MS/MS spectra of the top 10 most abundant ions were formed in the dark and the reaction quenched immediately by addition of the respective reducing reagent. The control sample was treated even-handedly but without any reduction and alkylation reagents. We then proceeded to tryptic in-solution digestion using FASP and SAX-fractionation into 6 fractions (8). Finally, we analyzed the fractions using LC-MS/MS. For each experimental condition, we performed 2 independent replicates and combined the data for Mascot database search.
ing using Proteome Discoverer. We defined products of the
different alkylation reactions at cysteine as variable modifica-
tion and calculated alkylation efficiencies, which were high for
all conditions, ranging between 97.01% (TCEP/CAA) and
99.84% (DTT/IAC), respectively (supplemental Table S2).
Despite the low variation in numbers of acquired MS/MS spectra
in the single experiments (coefficient of variation: 0.039,
supplemental Table S2), we observed strong differences in the
number of identified spectra between the different conditions.
The worst performance resulted from DTT in combination with
IAC (on average 19,054 peptidespectral matches, PSMs),
whereas DTT in combination with AA gave the best perform-
ance (29,390 PSMs) with an increase of 54% compared with
DTT/IAC (Fig. 2A, supplemental Table S2). We observed a
similar trend on the level of identified unique peptides (DTT/
IAC on average 8508 identifications and DTT/AA 11,690
identifications; corresponding to an increase of 37% Fig.
2B, supplemental Table S2) that was, however, less pro-
found compared with the PSM levels. In general, we ob-
served similar trends for the individual alkylation reagents
irrespective of the reducing reagent used. When we com-
pared the best alkylation conditions for each set, the differ-
ent reducing reagents only resulted in changes of up to 5%
on the PSM level. Samples alkylated with IAA and IAC
resulted in the lowest number of identified spectra with
DTT/IAC performing the worst. For DTT-reduced samples,
AA outperformed CAA, whereas the opposite was observed
for TCEP-reduced samples. For BME both AA and CAA
resulted in virtually the same number of identified PSMs.
Surprisingly, the control sample without any reduction and
alkylation yielded competitive results, identifying 84% of the
PSMs of the top condition DTT/AA (Fig. 2, supplemental
Table S2). Based on these results the best condition for
in-solution digestion of proteins is reduction of samples with
DTT in combination with alkylation using AA as it resulted in
the highest number of identified PSMs.

**Analysis of In-gel Digestion Data Sets**—One possible rea-
son for the discrepancies in identification rates are differences
in SAX fractionation performance of the in-solution digested
samples. We therefore generated another data set applying
the second strategy commonly used in proteomics sample
preparation: separation of proteins by SDS-PAGE, followed
by in-gel digestion (23). The sample buffer was omitting a
reducing reagent to exclude differential running behavior of
the proteins during electrophoresis associated to varying re-
duction efficiencies. We ran 50 μg of the cytosolic fraction on
SDS-PAGE gels (2 lanes for each condition) and cut the same
section of the gel, using ~20% of the lane, covering a region
between 30 to 50 kDa (supplemental Fig. S3). After reduction
and alkylation in the gel, samples were in-gel digested, ana-
lyzed by LC-MS/MS in 3 technical replicates, and the data
processed in the same way as the in-solution digested sam-
ple. We performed initial searches allowing for propionamide
at cysteine for all conditions to investigate for possible un-
wanted side reactions with free acrylamide in the gel. In all
conditions, except for AA as alkylating reagent, less than 1%
of the cysteine containing peptides were found to be propi-
onamide-modified. Therefore, propionamide at cysteine was
omitted in the following searches of samples not alkylated by
AA. Alkylation efficiencies were excellent for samples reduced
with DTT (for all alkylation reagents on average above 98.6%),
whereas samples reduced with BME and TCEP were slightly
lower. CAA in combination with TCEP, which resulted in av-
erage alkylation yields of only 95.2%, was the only exception
(supplemental Table S3). In contrast to the in-solution sam-
ple samples, the reduction reagent had a stronger influence on the

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**Fig. 1. A**, Experimental workflow for sample generation and analysis; **B**, reduction and alkylation reagents used; and **C**, combinations of reduction and alkylation reagents for generation of the different data sets. DTT: dithiothreitol, TCEP: tris(2-chlorethyl)-phosphate, BME: β-mercaptoethanol, IAA: iodoacetamide, IAC: iodoacetic acid, AA: acrylamide, CAA: chloroacetamide.
number of identified spectra. For IAA, AA, and CAA, BME clearly outperformed the other reduction reagents by almost doubling the number of PSMs identified on average in the case of IAA (DTT with 3193/11006 270 PSMs versus BME with 6281/11006 596 PSMs, Fig. 2C, supplemental Table S3). For the alkylating reagents, the iodine-containing reagents IAA and IAC performed worse than AA and CAA in all cases, irrespective of the reducing reagent. The biggest differences were observed in combination with DTT, where IAA treated samples resulted in 3193/11006 270 PSMs and CAA treated samples in 7365/11006 655 PSMs, respectively, resulting in an increase of 131%. Despite these large changes in PSMs, the number of acquired MS/MS spectra was roughly constant among all conditions (coefficient of variation: 0.028, supplemental Table S3). We observed similar trends for identified unique peptides (DTT/IAA 2435/11006 141 identifications and BME/CAA 4717/11006 637 identifications, corresponding to an increase of 94%, Fig. 2D). Best results were obtained for samples reduced with BME and alkylated with AA (8494/11006 767 PSMs). For the in-gel digests, the control sample, with neither reduction nor alkylation, performed worse than in the in-solution digested samples, identifying on average only 72% of PSMs resulting from the best condition BME/AA. It, however, still outperformed most of the conditions including the iodine-containing alkylation reagents (Fig. 2C, 2D, supplemental Table S3). We therefore recommend to reduce and alkylate samples for in-gel digestion by BME and AA.

Differences in Identification Efficiency—Next, we investigated possible reasons for the observed differences. Since the number of acquired MS/MS spectra did not change dramatically between conditions, we ruled out influences on protein digestion as possibility. We then investigated the efficiency of peptide identification (supplemental Fig. S4). For in-solution and in-gel digestion, the percentage of identified spectra varied considerably among the conditions, like the trends observed for PSMs. DTT/IAC resulted in the lowest efficiencies for both in-gel and in-solution digests (23 ± 2% identified spectra for in-gel digests and 37.5% identified spectra on average for in-solution digests). Highest efficiencies were obtained with BME/AA in-gel (50 ± 2% of the spectra identified) and BME/CAA in-solution (57.3% of the spectra identified on average). Among the evaluated alkylation reagents, IAA and IAC resulted in the lowest efficiencies of peptide identification across all conditions; indicating that the iodine-containing reagents lead to the highest numbers of non-specifically modified peptides. These results show that

![Fig. 2. Results of the analyses of 2 independent replicates of in-solution digested and SAX fractionated as well as 6 replicates of in-gel digested cytosolic fractions of HeLa cells using standard modifications for database searching. A, peptide spectral matches (PSMs) for in-solution digests; B, identified unique peptide sequences (UPSs) for in-solution digests; C, peptide spectral matches (PSMs) for in-gel digests; D, identified unique peptide sequences (UPSs) for in-gel digests. DI: DTT+IAA, DIA: DTT+IAC, DA: DTT+AA, DC: DTT+CAA, TI: TCEP+IAA, TIA: TCEP+IAC, TA: TCEP+AA, TC: TCEP+CAA, MI: BME+IAA, MIA: BME+IAC, MA: BME+AA, MC: BME+CAA.](image-url)
the reduction and alkylation conditions can have a strong effect on the identification efficiency of peptides.

Investigation of Cysteine-containing PSMs—As the alkylation reaction targets cysteine residues, we first investigated whether a change in the number of cysteine-containing peptides was the reason for the observed differences. For the in-solution digested samples (Fig. 3A left panel, supplemental Table S2), surprisingly, DTT in combination with IAA resulted in the highest number of identified cysteine-containing PSMs (8,178) despite its low performance related to the total number of identified PSMs. IAA also performed excellent for the other reducing agents (6601 PSMs for TCEP and 6694 PSMs for BME), whereas only TCEP/AA was better (7431 PSMs). For DTT and TCEP, CAA resulted in the lowest yields (5064 and 3789 PSMs, respectively), whereas IAC performed worst for the BME samples (2895 PSMs). With only 32 PSMs (out of 49,242 in total), the control sample resulted in virtually no identified cysteine-containing PSMs at all, indicating that all cysteine residues are present as disulfide bonds if the samples are not reduced and alkylated. The in-gel digested samples presented an entirely different result (Fig. 3A right panel, supplemental Table S3). Compared with DTT and

**Fig. 3.** A, Cysteine-containing PSMs identified in the in-solution and in-gel digested samples using normal search parameters; B, PSMs identified allowing for offsite alkylation at Y, S, D, T, H, E, K, and the peptide N terminus (single blots showing the abundance at each amino acid can be found in supplemental Figs. S6, S7); C, PSMs identified allowing for alkylation reagent dimers at Y, S, D, T, H, E, K, and the peptide N terminus (single blots showing the abundance at each amino acid can be found in supplemental Figs. S8, S9). Shown are the combined results from 2 independent replicates for the in-solution digested and 6 replicates for the in-gel digested samples, respectively. DI: DTT+IAA, DIA: DTT+IAC, DA: DTT+AA, DC: DTT+CAA, TI: TCEP+IAA, TIA: TCEP+IAC, TA: TCEP+AA, TC: TCEP+CAA, MI: BME+IAA, MIA: BME+IAC, MA: BME+AA, MC: BME+CAA, N-term: peptide N terminus.
Supplemental Tables S7, S8, as well as investigating \( b_2/a_2 \) ion fragmentation patterns (27), which is molecule (26) and are therefore only distinguishable when the \( b_2 \) ion during peptide fragmentation resulting in a single functional groups such as glycine, valine, or alanine, which were the alkylation reagents modify amino acids without functional groups such as glycine, valine, or alanine, which were found to be alkylated frequently, we evaluated the data manually. Almost all modifications of these amino acids were in the first two N-terminal amino acids. These amino acids form the \( b_2 \) ion during peptide fragmentation resulting in a single molecule (26) and are therefore only distinguishable when investigating \( b_2/a_2 \) ion fragmentation patterns (27), which is not considered in Mascot searches. We therefore assigned these alkylation products to the peptide N terminus, because it was most likely that the alkylation occurred at the N-terminal amino group, which has been shown to be efficiently alkylated (13). This resulted in a short-list of possibly modified residues (cysteine, the peptide N terminus, and 7 other amino acids), which were searched subsequently in single Mascot searches for 6 replicates of the in-gel digested samples and both replicates of the in-solution digests. We again detected alkylation at all specified amino acids with considerable differences between the alkylation reagents (Fig. 3B, supplemental Figs. S6, S7 as well as supplemental Tables S5, S6 for in-solution and in-gel digests, respectively). Irrespective of the digestion strategy, or the reagents used for reduction and alkylation, the peptide N terminus was modified most frequently and tyrosine least frequently. For in-solution digests, we identified the highest number of offsite alkylation with BME/IAC (3655 PSMs), followed by TCEP/AA (3132 PSMs) and DTT/IAC (2567 PSMs), whereas CAA resulted in the lowest number of unspecific modifications irrespective of the reducing reagent. Relative to the total number of assigned PSMs in each experiment, the in-gel digested samples resulted in significantly higher numbers of offsite alkylated residues compared with the in-solution digests. Particularly the iodine-containing reagents resulted in high numbers of offsite alkylation, which were on average between ~3- and ~10-fold higher when comparing individual iodine-containing to non-iodine-containing reagents. The lowest numbers were observed for TCEP in combination with AA (239 ± 84 PSMs), whereas DTT/IAC resulted in the highest numbers (2888 ± 375 PSMs). The highest occurrence of alkylation was found at the peptide N terminus followed by lysine, glutamic acid, and histidine residues for the iodine-containing alkylation reagents. Both, AA and CAA resulted in roughly similar numbers of offsite alkylation, whereas the N terminus, serine, threonine, and glutamic acid were modified most frequently. We mainly identified singly and doubly alkylated peptides, but also up to 6 times alkylated species were found (supplemental Fig. S10, supplemental Tables S5, S6). The identified offsite alkylation was not able to fully compensate for the discrepancies observed between the conditions. Therefore, we argued that additional modifications must occur during the alkylation process which were not identified with our current search strategy. It was shown that IAA can result in dimers at lysine residues mimicking the GlyGly tag found after tryptic digestion of ubiquitinated samples (19). We therefore performed searches for dimers of all 4 alkylation reagents at the 7 amino acids and the peptide N terminus analogous to the searches performed for the monomers. We identified double adducts at all amino acids for all alkylation reagents (Fig. 3C, supplemental Figs. S8, S9 and supplemental Tables S7, S8 for in-solution and in-gel digests, respectively). We also performed searches with iodination at both histidine and tyrosine (28), as it is possible that this reaction may occur when using iodine-containing reagents, but virtually no iodination was identified (data not shown). The high number of identified offsite alkylation shows impressively the reactivity of the chemicals used for alkylation at cysteine residues with basically every possible functional group present within a protein. Therefore, reduction and alkylation conditions should be chosen carefully employing minimal reagent concentrations and incubation times to minimize side reactions as efficiently as possible.

Quantification of Individual Peptide Losses by Dimethyl Labeling—The observed PSMs for each sample give an estimate how much of the sample was modified in general. They do, however, not allow calculating actual ratios for individual peptides. Since we frequently observed the modified and unmodified version of the peptides, we wondered to which degree those peptides were unintentionally modified. To investigate this, we applied a quantitative approach using dimethyl label-

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we observed some overlap between both alkylation strategies, in particular affected were several hundred significantly up- or down-regulated peptides. For IAA, surprisingly, the only amino acid strongly affected was methionine, being 3.8-fold over-represented in the downregulated peptides relative to the rest of the data set. Further analysis revealed that methionine was present in 252 out of the 315 peptides with a change of more than 2-fold in abundance. In the previous search for unspecific offsite alkylation, however, methionine was not found to be alkylated at all (supplemental Fig. S5). As the change in methionine-containing peptides may also be because of unexpected side reactions during dimethyl labeling, we investigated the in-solution and in-gel data sets for the abundance of each amino acid. We observed a depletion of methionine in both data sets when investigating the normalized amino acid composition across all identified peptides (Fig. 5B, C, supplemental Tables S2, S3), confirming the trend observed in the dimethyl data. We then plotted the numbers of methionine-containing PSMs to compare between the different conditions (Fig. 5D, supplemental Tables S2, S3). For both in-gel and in-solution digests, the iodine-containing alkylation reagents caused a strong reduction in the number of methionine-containing peptides with the lowest numbers of PSMs observed for the DTT/IAC treated sample (on average 2112 and 275 ± 25 methionine-containing PSMs for the in-solution and in-gel digested samples, respectively) and the highest for DTT/AA and for the in-solution digested samples (on average 8852 PSMs).

Analysis of Amino Acid Composition—Because both alkylation strategies add groups of differing molecular weight and the control sample is not alkylated at all, it is not possible to quantify the degree of unspecific alkylation at certain amino acids by investigating the offsite-alkylated peptides. Therefore, to investigate if a specific amino acid was most frequently affected, we analyzed the amino acid composition of peptides which changed in abundance more than 2-fold. We determined how frequently each amino acid occurred in the up- and down-regulated peptide groups of the AA and IAA treated sample relative to the control sample and normalized the determined amino acid composition to the whole data set (Fig. 5A, supplemental Table S9). In the data sets of AA alkylated samples for both the up- and down-regulated peptides we were not able to find any significant differences. For IAA, surprisingly, the only amino acid strongly affected was methionine, being 3.8-fold over-represented in the downregulated peptides relative to the rest of the data set. Further analysis revealed that methionine was present in 252 out of the 315 peptides with a change of more than 2-fold in abundance. In the previous search for unspecific offsite alkylation, however, methionine was not found to be alkylated at all (supplemental Fig. S5). As the change in methionine-containing peptides may also be because of unexpected side reactions during dimethyl labeling, we investigated the in-solution and in-gel data sets for the abundance of each amino acid. We observed a depletion of methionine in both data sets when investigating the normalized amino acid composition across all identified peptides (Fig. 5B, C, supplemental Tables S2, S3), confirming the trend observed in the dimethyl data. We then plotted the numbers of methionine-containing PSMs to compare between the different conditions (Fig. 5D, supplemental Tables S2, S3). For both in-gel and in-solution digests, the iodine-containing alkylation reagents caused a strong reduction in the number of methionine-containing peptides with the lowest numbers of PSMs observed for the DTT/IAC treated sample (on average 2112 and 275 ± 25 methionine-containing PSMs for the in-solution and in-gel digested samples, respectively) and the highest for DTT/AA and for the in-solution digested samples (on average 8852 PSMs) and BME/AA for the in-gel digested samples (2632 ± 209.
PSMs). These data clearly indicate that methionine became efficiently modified by the iodine-containing alkylation reagents IAA and IAC, but not by the non-iodine reagents AA and CAA. Therefore, a covalent modification induced by the presence of iodine-containing alkylation reagents, should have been identified in the MASCOT searches at the side chain of methionine.

Error and Mass Tolerant Searches—Because we were not able to identify any methionine-containing peptides with alkylated side chains (supplemental Fig. S5) we performed error tolerant (29) and mass tolerant (30) searches to investigate for the missing methionine-containing peptides using two independent replicates of the in-gel digested samples (supplemental Table S10). In the error tolerant searches, no modifications that have not already been found in our other database searches were identified in significant numbers (data not shown). For the mass tolerant searches, we initially also did not observe any striking differences between the treatments despite a higher incidence rate of identified peptides with a mass shift of +57 Da, which is because of the
offsite alkylation mentioned above (Fig. 6A). When we repeated the mass tolerant searches allowing for oxidation of methionine, however, we observed more than 1000 PSMs for the samples alkylated with iodine-containing alkylation reagents at \(-64\) Da (Fig. 6B), which were not present in the samples alkylated with AA and CAA. These PSMs contained methionine. The neutral loss of 64 Da from the molecular peptide ion is defined as the loss of the side chain from oxidized methionine residues (www.unimod.org and (31, 32)). When oxidation at methionine is allowed as variable modification in the Mascot searches, the neutral loss of 64 Da is considered for annotation of the fragment ions. This explains why these spectra were only identified in searches with oxidation at methionine and not already in the other searches at a mass deficiency of 48 Da, as it should make no difference in the mass tolerant searches whether a mass error of 64 or 48 Da is assigned. Without the modification and the assignment of a neutral loss of 64 Da to fragment ions, however, \(-50\%\) of the fragment ions in the MS/MS spectra could not be matched by Mascot resulting in low scoring peptide identifications falling below our applied score cutoff of 30. These searches showed that there was indeed a modification at methionine residues which results in a loss of the side chain of the amino acid.

**Investigation of Methionine Side Chain Loss Using a Synthetic Peptide**—To further investigate the side chain loss induced by iodine-containing alkylation reagents, we incubated the synthetic peptide APEIMLNSK with DTT, followed by IAA, IAC, AA, and CAA, and analyzed the samples with MALDI-MS (Fig. 7A). For the samples treated with IAA and IAC a strong loss of the methionine side chain from the molecular ion \((-48\) Da) could be observed. AA and CAA did not result in any losses, though we noticed offsite alkylation of a fraction of the peptides \((-25\%\) for the AA treated and \(~10\%\) for the CAA treated sample). Fragmentation of the \([\text{M}-48+\text{H}]^+\) of the IAA-derivatized peptide clearly proved that the loss indeed originated from the methionine residue (Fig. 7B). To determine if the loss of the side chain exclusively depends on the alky-
A prominent loss of the side chain of methionine can be observed from the molecular ion (M+H)⁺ for the peptides alkylated using iodine-containing reagents; B, MS/MS spectrum of the [M+H]⁺ of the unmodified peptide (upper panel) and the signal at M-48Da (lower panel). The fragment ions show that the loss of 48 Da originates from the methionine side chain.

Investigation of Methionine Side Chain Loss in LC-MS/MS Measurements—We observed the peptide lacking the methionine side chain in the MALDI measurements of the alkylated peptides, but not its alkylated form. Furthermore, we only observed peptides in the mass tolerant searches, which already had lost the side chain of methionine. Therefore, the loss of the methionine side chain, in principle, can be either because of an elimination in-solution, or because of an efficient in-source fragmentation of carbamidomethylated (IAA) or carboxymethylated (IAC) methionine. It was previously described, that peptides containing carbamidomethylated methionine can undergo in-source fragmentation at high cone voltages, while staying intact at lower values (17). In another study, it was shown that carbamidomethylated methionine can cause a neutral loss of 105 Da when fragmented in the parent ion scanning mode further indicating the presence of intact carbamidomethylated methionine in ESI ionization (18). As the searches allowing for carbamidomethyl at methionine did not yield any PSMs in our data sets (supplemental Fig. S5) we defined a modification accounting for the loss of the side chain (-SHCH₃) for identification of MS/MS spectra from in-source fragmented peptides, and performed Mascot searches. For the in-solution digested samples we identified up to 703 PSMs lacking the side chain of methionine (DTT/IAC) and up to 198 ± 101 (BME/IAC) for the in-gel digested samples.
samples (supplemental Fig. S15, supplemental Tables S11 and S12). When we investigated the *.raw files we observed a prominent peak at +105 (for IAA) and +106 (for IAC) Da, which coeluted with the peptides annotated with a loss of the methionine side chain (Supplemental Figs. S16, S17). The MS/MS fragment ion spectra of these peaks showed a prominent peak corresponding to the neutral loss of the alkylated methionine side chain but otherwise no fragment ions were observed from which any sequence information could be derived (supplemental Fig. S18). We therefore defined multi stage activation (MSA, (33)) methods defining the loss of the modified side chain for the individual alkylation reagents as a neutral loss for triggering the MSA scan. We then remeasured the samples of one replicate of the in-gel digests treated with DTT and all alkylation reagents with the respective MSA method. Subsequently, we performed database searches allowing for alkylation of methionine (MS/MS or MSA of intact peptides), carbamylation of methionine (MS/MS of intact peptides) and methionine oxidation (Fig. 8, supplemental Table S13). For IAA treated samples, 19% of the identified methionine-containing PSMs were unmodified, 31% oxidized, and 50% carbamidomethylated (sum of PSMs identified for MSA and MS/MS of in-source fragmented peptides), whereas for IAC only 6% were unmodified, 43% oxidized, and 51% carboxymethylated (sum of PSMs identified for MSA and MS/MS of in-source fragmented peptides). For AA and CAA 63 and 64% of the methionine-containing peptides were unmodified, respectively, whereas 32 and 33% were oxidized and only negligible amounts were annotated to be alkylated. We conclude therefore that the alkylated side chains of methionine were predominantly formed by iodine-containing reagents (as already indicated by the MALDI measurements) and that they were stable in-solution. In the gas phase, however, they fragmented efficiently either in the source of the mass spectrometer or during targeted MS/MS fragmentation resulting in an abundant neutral loss and preventing identification of the peptide.

**DISCUSSION**

In the current study, we show that reduction and alkylation conditions can have a strong impact on the identification of peptides because of unspecific alkylation using an acetone precipitated cytosolic fraction of HeLa cells, re-solubilized in 0.1 M TRIS-HCl/4% SDS at 95 °C. Interestingly, for in-gel and in-solution digestion of proteins, different combinations of reduction and alkylation reagent were found to give the best results. For the reducing reagents, surprisingly, TCEP and BME, the substances used least frequently by the community (in 21 and 0% of articles investigated, respectively, supplemental Fig. S1, supplemental Table S1), resulted in highest numbers of peptide and protein identifications for the in-gel digests treated with DTT and all alkylation reagents with the respective MSA method. Subsequently, we performed database searches allowing for alkylation of methionine (MS/MS or MSA of intact peptides), carbamylation of methionine (MS/MS of intact peptides) and methionine oxidation (Fig. 8, supplemental Table S13). For IAA treated samples, 19% of the identified methionine-containing PSMs were unmodified, 31% oxidized, and 50% carbamidomethylated (sum of PSMs identified for MSA and MS/MS of in-source fragmented peptides), whereas for IAC only 6% were unmodified, 43% oxidized, and 51% carboxymethylated (sum of PSMs identified for MSA and MS/MS of in-source fragmented peptides). For AA and CAA 63 and 64% of the methionine-containing peptides were unmodified, respectively, whereas 32 and 33% were oxidized and only negligible amounts were annotated to be alkylated. We conclude therefore that the alkylated side chains of methionine were predominantly formed by iodine-containing reagents (as already indicated by the MALDI measurements) and that they were stable in-solution. In the gas phase, however, they fragmented efficiently either in the source of the mass spectrometer or during targeted MS/MS fragmentation resulting in an abundant neutral loss and preventing identification of the peptide.
the identification of cysteine-containing peptides. This may be because of the removal of TCEP before adding the alkylation reagents. It is common practice to perform reduction and alkylation in the same step when using TCEP as it was the case in our in-solution digests (because we did not remove TCEP before addition of the alkylation reagent). It may therefore increase performance for in-gel digested samples if a mixture of TCEP and the alkylation reagent is used. For the experimental setup used in this study, we recommend BME for reducing proteins in SDS-PAGE gels. For in-solution digests, DTT, BME, and TCEP performed equally well and the choice should be made based on potential preferences in the alkylation reagents as AA seems to work better with DTT and BME, whereas CAA performs better in combination with TCEP. This was especially the case regarding the alkylation efficiency of cysteine-containing peptides, which may introduce inconsistencies, for example, in peptide quantification. In general, the iodine-containing alkylation reagents (IAA and IAC) resulted in the worst identification rates irrespective of the digestion condition, or reducing reagent, and should therefore be avoided. We compared in this study alkylation reagents that are structurally related. There are, however, also other iodine-free reagents, such as N-ethylmaleimide (NEM), methyl methanethiosulfonate (MMTS), or 4-vinylpyridine, which are structurally not related. These compounds will probably also result in lower numbers of side reactions compared with the iodine-containing reagents.

Surprisingly, for both digestion strategies, the samples that were neither reduced nor alkylated resulted in good numbers of spectra and protein identifications despite the total lack of cysteine-containing peptides. This shows that disulfide bridges do not seem to hamper the proteolytic cleavage by trypsin significantly as we also could not see an increase in missed cleavage sites in these samples (data not shown). The reproducibility between the single replicates for the untreated samples ranged among the highest compared with the other conditions, further exemplifying the negative effect of random unspecific modifications on the quality of sample preparation. The complete lack of cysteine-containing peptides in these data sets indicated an efficient formation of disulfide bridges among proteins or peptides and underlines the necessity of efficient reduction and alkylation in sample preparation for proteomics experiments.

For the in-solution digested samples the percentage of identified, nonspecifically modified peptides was lower and alkylation efficiencies better compared with the in-gel digested samples. This is most likely because of the more efficient removal of chemicals by FASP and the fact that the reduction and alkylation chemicals must diffuse into the gel pieces, which may alter accessibility of the proteins. Reduction and alkylation before gel electrophoresis would therefore most likely improve reproducibility, as it allows for better accessibility of the proteins and immediate separation of the proteins from the reactive chemicals during electrophoresis. Over all, we observed significantly higher differences in spectra identification rates between the alkylation reagents in the in-gel digests compared with the in-solution digests. There are several factors that may be responsible for this: (1) we used higher concentrations for the alkylation reagents in the gel (20 mM for the in-solution digests and 55 mM for the in-gel digests); (2) the alkylation reactions were quenched by addition of the respective reducing reagents for the in-solution digests, but not the in-gel digests; (3) because of the FASP strategy applied for the in-solution digests reactive chemicals were removed immediately after performing the reaction. Therefore, for the in-gel digests, unspecific reactions could proceed for a longer time, which also explains the higher degree of modified peptide N termini in the data set as this can only happen after tryptic cleavage. These factors influence the reaction kinetics of the alkylation reactions which is probably a major reason for the differences observed. If other protocols are applied (e.g. by different reagent conditions, altered incubation times, or different buffer systems) the differences observed may differ significantly. Additionally, the in-solution digests were analyzed with 30 min gradients whereas the in-gel digests were analyzed with 60 min gradients. This resulted in ~100,000 spectra for the whole in-solution data set and ~35,000 spectra for the in-gel data set, respectively. As the in-gel data set was only derived from ~1/5 of the sample the average instrument time per number of peptides contained in the sample was higher by a factor of almost 2-fold, making it more likely to fragment and identify low abundant species in the in-gel digests. For SAX on the other hand, because of incomplete separation of highly abundant unmodified peptides between the different fractions, the effect of the alkylation reagents might have been masked because the low abundant derivatized peptide species were not fragmented at all.

When we investigated the data sets for unwanted side reactions, searches of the in-solution digests, considering multiple variable modifications, produced in almost all cases lower numbers of PSMs compared with the searches without offsite alkylation, which we performed initially. This was most likely because of the increased cutoff for peptide acceptance in order to maintain an FDR of 1% with the higher numbers of variable modifications (and therefore a strong increase in search space). This leads to lower total numbers of PSMs, even though a high number of modified peptides is identified. Therefore, it is difficult to assess how far the identified offsite alkylation can compensate for the lack of identified PSMs in the initial data sets of the in-solution digests. For the in-gel digests, searching for offsite alkylation resulted in greatly increased numbers of peptide identifications for the iodine-containing reagents almost reaching PSM numbers of the iodine-free alkylation reagents. For the latter, however, including the offsite alkylation in the searches always reduced the number of identified peptides presenting a similar picture as for the in-solution digests. For the samples alkylated with
iodine-containing reagents, the searches including offsite alkylation where never able to match the numbers of unique peptide sequences obtained in the searches without any offsite alkylation. Even though resulting in almost twice the numbers of PSMs they stayed significantly below the iodine-free alkylation reagents. This is probably because of high abundant modified peptides preventing fragmentation of lower abundant unmodified species. It is therefore in our eyes not reasonable to continue using iodine-containing alkylation reagents and trying to compensate for their unspecific side reactions by altering the database search strategy.

When we investigated identification efficiencies in our data sets, we realized that it was possible to increase identification rates tremendously for the in-gel digests (from 24% in case of DTT/IAA to 54% for BME/CAA). Given that currently the majority of laboratories still work with DTT/IAA and it was recently estimated that on average only 25% of the spectra are identified in proteomics experiments (34) it may be sufficient to simply change reduction and alkylation conditions to increase identification efficiency in proteomics experiments considerably.

Surprisingly, one of the amino acids which was affected most in our data sets was methionine. So far, in studies which dealt with the investigation of unspecific modifications by alkylation in proteomic sample preparation, alkylated methionine was not detected at all. In Unimod (www.unimod.org), which is a database containing modifications used for database searching in proteomics experiments, carbamidomethylation or carboxymethylation at methionine is not even defined. To our knowledge, there are only 2 studies which mentioned alkylation at methionine and in both the alkylation was performed with IAA. One dealt with the analysis of an intact protein (17) and determined the mass of the carbamidomethylation at methionine to be 58 Da, whereas the second study was focused on the analysis of peptides (18) and identified the modification to be 57 Da. We confirmed in our experiments the mass value of 57 Da. Interestingly, we had the impression that the iodine-containing alkylation reagents seem to preferentially alkylate methionine in the synthetic peptide we used rather than the peptide N terminus, which we found to be alkylated most frequently in other peptides. In the experiments with the synthetic peptide, we only found the methionine alkylated peptide (resulting in a neutral loss of 105 Da or 48 Da relative to the unmodified molecular ion) but no additional offsite alkylation on this peptide at all. Neither on the one alkylated at the methionine, nor on the non-alkylated in the same sample. If we, however, oxidized the methionine in the peptide with H2O2 before performing the alkylation reaction, we observed prominent offsite alkylation at other residues. It therefore seems that the alkylation at methionine prevents the other residues in the peptide to be alkylated because we applied the alkylation reagent in a vast excess for this experiment and depletion of the reagent by the methionine residues is therefore highly unlikely. Along this line, also in the general data sets, we observed in the majority singly offsite alkylated peptides. This could be because of a change in the charge of the peptide or maybe interactions of the alkylated residue with other side chains that would usually be susceptible to modification.

CONCLUSION

Our systematic comparison of reduction and alkylation procedures shows that the reagents used can have a strong impact on the outcome of a proteomic experiment. Reduction reagents do have an effect; it is, however, rather moderate compared with the alkylation reagents as only the latter result in unspecific modifications of amino acids. Basically, all functional groups can react with the used alkylation reagents with different efficiencies. Especially iodine-containing reagents are prone to unspecific side reactions and should be completely avoided. The non-iodine-containing reagents result in similar cysteine alkylation efficiencies but markedly increased performance in the identification of peptides. To maximize performance and minimize unwanted side reactions, we suggest the usage of DTT and AA for in-solution digests and BME with AA for in-gel digests.

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DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (35) partner repository (https://www.ebi.ac.uk/pride/archive/) with the data set identifier PXD005183.

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† To whom correspondence should be addressed: Institute for Biochemistry and Molecular Biology, University of Bonn, Nussallee 11, 53115 Bonn, Germany. Tel.: +49 228 737081; Fax: +49 228 732416; E-mail: dominic.winter@uni-bonn.de.

§ Current affiliation of TM: German Cancer Research Center, Heidelberg, Germany.

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