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Mass Spectrometry of Intact Proteins Reveals +98 u Chemical Artifacts Following Precipitation in Acetone

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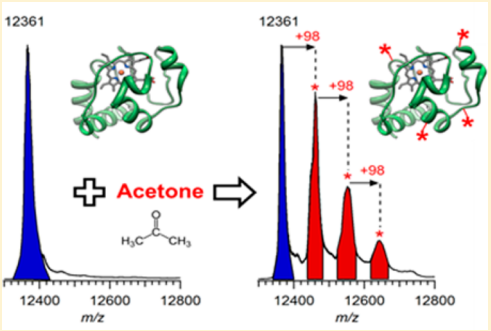
Supporting Information

ABSTRACT: Protein precipitation in acetone is frequently employed ahead of mass spectrometry for sample preconcentration and purification. Unfortunately, acetone is not chemically inert; mass artifacts have previously been observed on glycine-containing peptides when exposed to acetone under acidic conditions. We herein report a distinct chemical modification occurring at the level of intact proteins when incubated in acetone. This artifact manifests as one or more satellite peaks in the MS spectrum of intact protein, spaced 98 u above the mass of the unmodified protein. Other artifacts (+84, +112 u) also appear upon incubation of proteins or peptides in acetone. The reaction is pH-sensitive, being suppressed when proteins are exposed to acetone under acidic conditions. The +98 u artifact is speculated to originate through an intermediate product of aldol condensation of acetone to form diacetone alcohol and mesityl oxide. A +98 u product could originate from nucleophilic attack on mesityl oxide or through condensation with diacetone alcohol. Given the extent of modification possible upon exposure of proteins to acetone, particularly following overnight solvent exposure or incubation at room temperature, an awareness of the variables influencing this novel modification is valued by proteomics researchers who employ acetone precipitation for protein purification.



KEYWORDS: *protein modification, acetone, 98 u, aldol, diacetone alcohol, mesityl oxide, mass artifact*

INTRODUCTION

Protein precipitation is routinely employed for small or large-scale sample purification,[1] being favored ahead of proteome analysis by gel electrophoresis, and liquid chromatography–mass spectrometry. Numerous options exist to induce precipitation, including altering the solution temperature[2] or pH,[3] through addition of salts,[4] polymers,[5] or organic solvents.[6] An optimal precipitation strategy reliably recovers purified proteins in high yield but without altering the sample, as would occur through unintentional chemical modifications. Such artifacts would propagate throughout the detection workflow and ultimately impact the success of sample characterization.

With potential for exceptional recovery and purity, organic solvent precipitation has steadily risen in popularity as a front-end tool for proteome analysis. Several groups have demonstrated the merits of acetone precipitation in bottom-up and top-down proteomics workflows. The importance of ionic strength in maximizing protein recovery with acetone precipitation has been noted.[7] Under the proper conditions, near-quantitative recovery is obtained with high repeatability, including for dilute protein samples.[7,8] Puchades et al. evaluated acetone precipitation for SDS depletion ahead of MALDI MS analysis, reporting a 100-fold reduction of SDS.[9] Our group extended acetone precipitation to SDS depletion ahead of LC-ESI-MS/MS analysis.[10,11] Kelleher employed acetone precipitation for SDS depletion in top-down[12] and middle-down[13] proteomic workflows. The approach outperforms the popular FASP method for SDS removal,[14] affording greater identification of peptides by MS.[15] It has been demonstrated that acetone-precipitated proteins initially containing SDS provide proteome sequence coverage superior to that of an equivalent tryptic digest of the nonprecipitated, surfactant-free proteome mixture.[15,16]

Though analyte recovery and purity are important considerations of protein sample preparation, one must also consider *in vitro* protein modifications induced as artifacts of sample handling. Modifications are common to several proteomic workflows, including acrylamide adduct formation during SDS-PAGE,[17] methylation by methanol,[18] oxidation via electrospray,[19] glycerol esterification,[20] urea carbamylation,[21] and formylation by formic acid.[22,23] These artifacts increase sample complexity and challenge quantitative analysis, particularly if the mass shift matches that of *in vivo* protein modification.[24] If the modifications are known, then steps can be taken to minimize the reaction; alternatively, the resulting mass shifts can be accounted for during data analysis. Unknown chemical modifications are detrimental to proteome analysis, as the unaccounted mass shifts will preclude proper MS identification.

Despite its long history,[25] it was only recently reported that acetone precipitation may manifest artifacts in the resulting MS spectra. Acetone is not chemically inert; 1° amines are reactive toward ketones to yield imines.[26,27] A reaction with acetone at

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lysine or the N-terminus of a protein or peptide would form a ketimine, with a hypothetical mass shift of +40 u. Schiff base formation is pH-sensitive, being labile under the acidic conditions typically encountered during LC-ESI/MS. However, as reported by Simpson et al., a +40 u artifact was observed in the mass spectrum of peptides exposed to traces of acetone under acidic conditions.[28] The modified peptides contained glycine at the second position and account for some 5% of the total proteome, though the exact nature of this chemical reaction was not determined.[28] To date this is the only reported unintentional modification of peptides or proteins induced by acetone. Still other reactions with acetone may be possible. Aldol condensation of acetone forms mesityl oxide (M_r 98 u) which proceeds though the intermediate diacetone alcohol (M_r 116 u).[29] Each of these compounds may in turn react with proteins. Aldolases for example are enzymes which catalyze the formation or breakdown of aldols through a Schiff base intermediate at a catalytic lysine center.[30] Again, one notes the reversible nature of this reaction under acidic conditions, implying that any potential modification may not manifest in the resulting mass spectrum.

While investigating intact proteins precipitated in acetone through ESI-MS, we observed multiple satellite ions spaced +98 u from the expected protein signal. We herein report such mass shifts to originate from a novel chemical modification induced by acetone. The extent of protein modification is time-, temperature-, and pH-sensitive, being suppressed under acidic conditions. However, once formed the modification is stable to acidic environments. We discuss the origins of this novel type of acetone modification as well as its implications in proteome analysis workflows.

MATERIALS AND METHODS

Chemicals

Cytochrome c, myoglobin, ubiquitin, bovine serum albumin (BSA), hemoglobin, trypsin, mesityl oxide, diacetone alcohol, MS-grade trifluoroacetic acid (TFA), and formic acid were purchased from Sigma-Aldrich (Oakville, CA). Model peptides were purchased from GL Biochem Ltd. (Shanghai, China). HPLC-grade acetone, acetonitrile, and methanol were from Fisher Scientific (Ottawa, CA). Reagent-grade glacial acetic acid was from Caledon Laboratory Chemicals (Georgetown, CA), while reagent-grade ammonium hydroxide (28% solution) was from ACP Chemicals Inc. (Montreal, CA). Chemicals used for digestion, including urea, dithiothreitol (DTT), and iodoacetamide (IAA), were from Bio-Rad (Mississauga, CA). Throughout the study, Milli-Q purified water (18.2 MΩ cm) was used.

Preparation of Apo-Cytochrome c

Removal of heme from cytochrome c was achieved by a modification of the method of Fisher et al.[31] To 60 μL of 10 mg/mL cytochrome c, 1 mg of $AgNO_3$ in 113 μL of water and 10 μL of acetic acid was added. The mixture was incubated in the dark for 4 h at 40 °C then centrifuged to pellet the heme (15 min, 10 000 × *g*). The protein in the supernatant was purified by HPLC using a self-packed 10 cm × 1 mm POROS R2 column (20 μm beads, Applied Biosystems) and collected as a single fraction by a rapid solvent ramp of acetonitrile from 5 to 50%. The solvent was evaporated in a SpeedVac vacuum concentrator, and the protein was dissolved in 100 μL of 50 mM ammonium acetate (pH 5.0), which contains 6 M guanidine HCl and 1 M DTT. The solution was incubated at room temperature for 2 h in the dark and then centrifuged (15 min, 10 000 × *g*). The supernatant was again injected onto a POROS R2 column, collecting the purified apo-cytochrome c as a single fraction, wherein the protein has no measurable absorbance at 410 nm.

Protein Precipitation

Standard protein stock solutions were prepared in water from lyophilized powder to a concentration of 1 g/L. Peptide samples were prepared in water to 100 μM. Working solutions of the proteins were diluted to 0.1 g/L in water or water with one of the following pH adjusters: 0.1% TFA, 0.1% formic acid, 0.1% acetic acid, 50 mM ammonium bicarbonate, 50 mM Tris-HCl (pH 8), 10 mM NH_4OH, or 2 M NH_4OH. The sample was then mixed with four volumes of ice-cold acetone and incubated at a specified temperature (−20 °C, unless otherwise indicated) for a defined period (1 h, unless otherwise stated). Samples were then fully dried in a SpeedVac concentrator.

Proteins were also precipitated in methanol, acetonitrile, or acetonitrile containing either 0.1% (v/v) mesityl oxide or 0.1% diacetone alcohol. The conditions for precipitation in these solvents, including protein concentration, solvent ratio, temperature, and time, are as described above.

Escherichia coli Proteome Extraction and Precipitation

E. coli was inoculated into LB media and grown overnight at 37 °C with shaking according to the standard protocols.[32] Cells were harvested at an OD_{600} of 1.0 by centrifugation (15 min, 5000 × *g*), then washed with PBS buffer, and finally washed with water. The cells were suspended in 50 mM Tris-HCl buffer (pH 8.0), then snap-frozen in liquid nitrogen and ground via mortar and pestle. The resulting cell lysate was heated to 95 °C for 5 min, then centrifuged (30 min, 16 000 × *g*, 4 °C). The protein content in the supernatant, as measured using BCA assay (Pierce, Rockford, IL), was adjusted in Tris buffer (pH 8) to a final concentration of 0.5 g/L. A 100 μL aliquot of the extracted *E. coli* proteins was subjected to acetone precipitation protocol as described above (4:1 volume ratio, −20 °C, 1 h).

Protein Digestion

A 50 μg portion of *E. coli* proteome or a 50 μg portion of BSA was subjected to trypsin digestion following precipitation. The pellet was resuspended in 20 μL of 8 M urea with repeat pipetting followed by 5 min sonication to aid in sample dispersion. Next, 80 μL of 50 mM ammonium bicarbonate was added, and proteins were reduced through addition of 4.75 μL of 200 mM DTT (20 min, 60 °C), then alkylated with 10.5 μL of 200 mM IAA (20 min, 20 °C). Digestion occurred following addition of 1 μg of trypsin to the *E. coli* extract, or 2.5 μg of trypsin for BSA, with incubation at 37 °C overnight. Digestion was terminated through addition of 10% TFA; then, peptides were desalted by reversed phase HPLC cleanup using a self-packed 10 cm × 1 mm C18 column (5 μm beads, Waters, Mississauga, CA) and collected as a single fraction by a rapid solvent ramp of acetonitrile from 5% to 85%.

LC-MS Analysis of Intact Proteins or Peptides

Following precipitation, the dried protein pellets were resolubilized in 100 μL of 15% acetonitrile and 0.1% formic acid in water (15 min, ultrasonication bath). The entire sample was immediately injected onto a self-packed 30 cm × 0.5 mm POROS R2 column coupled to an Agilent 1200 HPLC system. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. The following gradient was employed: 0 min, 15% B; 5 min, 15% B, 5.01 min, 50% B; 15

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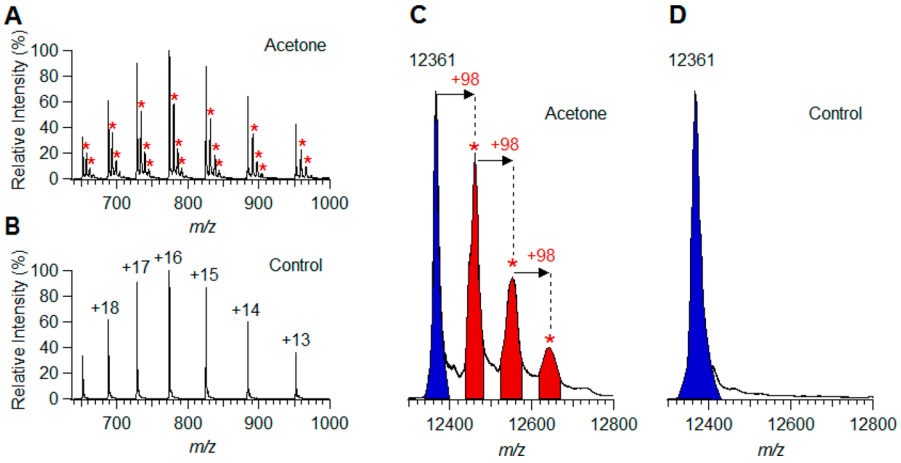

Figure 1. ESI-MS spectra of cytochrome c showing appearance of +98 u satellite peaks. (A) Artifacts are observed following precipitation in 80% acetone (−20 °C, overnight) which are absent from the nonprecipitated control, (B). (C) and (D) Equivalent samples are presented as deconvoluted MS spectra (cytochrome c M_r 12 361 u).

min, 50% B; 15.01 min 15% B. The solvent flow rate (100 μL/min) was split postcolumn to direct 10 μL/min to the ESI source of a ThermoFisher LTQ linear ion trap mass spectrometer (San Jose, CA). Data acquisition was in positive MS-only mode, scanning over the range m/z 400−2000. For exact mass determination of the mass adduct on intact protein, acetone-modified cytochrome c was subjected to analysis on an Orbitrap Velos Pro (ThermoFisher Scientific), employing self-packed 30 cm × 75 μm spray tips (New Objective, Woburn, MA) containing 4 μm C12 beads (Phenomenex, Torrance, CA) and coupling to nanospray at a flow rate of 0.25 μL/min. The isocratic solvent system was 50% acetonitrile in water with 0.1% formic acid. Peptide experiments (MS and MS/MS) were conducted on an LTQ XL linear ion-trap mass spectrometer. The peptides were further analyzed on an Orbitrap Exactive mass spectrometer for exact mass determination of the adduct observed on peptides. The samples were introduced into the ESI source of both instruments via syringe pump and the systems were operated in the positive mode, scanning over the mass range m/z 150−2000.

The data obtained for intact protein from Orbitrap Velos Pro was deconvoluted using Xcalibur software which accompanies the Orbitrap platform, whereas other data for intact protein were deconvoluted using Microsoft Excel. MS spectra of intact proteins and peptides were plotted using Igor Pro Software package (WaveMetrics, Lake Oswego, OR).

LC-MS/MS of Tryptic Peptides and Database Search

For BSA, 1 pmol of desalted peptides was analyzed on the LTQ linear ion trap mass spectrometer using dual capillary LC-MS/MS system,[33] employing self-packed 30 cm × 75 μm spray tips (New Objective, Woburn, MA) containing 4 μm C12 beads (Phenomenex, Torrance, CA) and coupling to nanospray at a flow rate of 0.25 μL/min. The same solvent system was employed as for intact protein analysis, through with the following gradient: 0 min, 5% B; 0.1 min, 7.5% B; 45 min, 20% B; 57.5 min, 25% B; 60 min, 35% B; 61 min, 80% B; 64.9 min, 80% B; 65 min, 5% B. The LTQ operated in data-dependent mode (MS followed by MS/MS of top three peaks) with 30 s dynamic exclusion. The *E. coli* digest was analyzed on an Orbitrap Velos Pro (ThermoFisher Scientific). The desalted peptide digest (1 μg) was injected onto a self-packed 15 cm × 0.1 mm monolithic C18 column (Phenomenex) using a Dionex Ultimate 3000 Rapid Separation LC nanosystem (Bannockburn, IL). The solvent gradient was as follows: 0 min, 3% B; 3 min, 3% B; 5 min, 5% B; 69 min, 35% B; 72 min, 95% B; 77 min, 95% B; 80 min, 3% B. The LC column was coupled to a 10 μm New Objective PicoTip noncoated Emitter Tip (Woburn, MA). The Orbitrap operates at a resolution of 30 000 fwhm in MS mode, scanning in data-dependent mode (MS/MS of top ten peaks) at a scan rate of 66 666 u/s, <0.6 u fwhm. Dynamic exclusion was applied for 25 s over a range of ±5 ppm. MS/MS data were searched using Proteome Discoverer software against either the *E. coli* K12 UniProt database (downloaded on May 2014, 4269 entries) with a mass tolerance of 3 ppm (high-resolution MS mode) and 0.8 u (MS/MS mode) or a BSA sequence with a mass tolerance of 0.8 u (low-resolution MS mode) and 1 u (MS/MS mode). The searches allowed for up to two missed cleavages, assigning a peptide false-positive rate of 1% and minimum two peptides per protein. Peptide modifications included oxidation of methionine and carbamidomethylation at cysteine. Protein modification caused by incubation in acetone was first searched by assigning +98.0732 u dynamic modification to all amino acid residues. The data were searched again, confining +98.0732 u to histidine, lysine, or arginine residues. Finally, data were searched for a +40 u dynamic modification on all amino acids as well as the N-terminus.

RESULTS

Acetone precipitation of proteins is favored for SDS depletion ahead of MS analysis. In the deconvoluted MS spectrum, the presence of adducts spaced by 266 u is indicative of incomplete detergent removal. Our lab also routinely monitors potential formylation (+28 u), given our use of cold (−20 °C) formic acid to resolubilize acetone-precipitated protein.[34] Performed properly, an acetone-precipitated protein would be free of these and other adducts, maximizing MS signal quality, reducing spectral complexity, and also improving chromatographic performance. Obtaining a high-quality MS spectrum is critical to the success of top-down proteomics workflow.

Through characterization of intact proteins by ESI-MS, we occasionally observe a set of satellite peaks, successively spaced 98 u above the mass of the unmodified protein. Exact mass determination of the modification reveals a $\Delta m/z$ of 98.08 ± 0.01 u (see Figure S1 for a sample high-resolution MS

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spectrum). A second, less intense series of ions are spaced at 84 u (exact mass 84.06 ± 0.01 u). These series of ions correlate with inclusion of acetone for protein precipitation. Satellite peaks are observed for samples precipitated with or without SDS or formic acid to resolubilize the sample. Nonprecipitated proteins are generally free of +98 u artifacts, as are samples precipitated with other organic solvents (methanol or acetonitrile, see Figure S2). The +98 u artifacts were consistent across multiple batches of acetone, on different MS platforms, and across multiple laboratories. Figure 1 depicts the ESI-MS spectrum of cytochrome c following incubation in acetone (Figure 1A) relative to the control (Figure 1B). The deconvoluted spectrum (Figure 1C) depicts the intense satellite ion series spaced successively at +98 u. These multiple peaks impact the overall signal intensity and lower spectral quality. In this figure, the artifacts account for 56% of the total protein signal. Considering the added sample complexity, the absolute intensity of the unmodified cytochrome c represents only 20% of the nonprecipitated control.

In localizing the origin of the +98 u MS artifacts, we first considered the potential formation of adducts of either sulfates ($M + H_2SO_4$, $\Delta m/z$ 97.967) or phosphates ($M + H_3PO_4$, $\Delta m/z$ 97.977) as contaminants of the system.[35,36] The difference between the expected mass shift of these adducts to that observed on an Orbitrap ($\Delta m/z$ 98.08 ± 0.01) was over 1000 ppm, which excludes these as possible origins of the adduct. In addition, as initially reported by Chait, it was suggested that these weakly associated ion complexes can be removed via in-source dissociation.[36] Attempts to reduce of the intensity of the +98 u ions in this manner were unsuccessful; further varying the CID collision energy on a linear ion trap mass spectrometer also failed to eject neutral adducts and ultimately resulted in fragmentation of the full protein (not shown).

Analysis of acetone-precipitated proteins by liquid chromatography coupled to ESI-MS also confirms the presence of +98 u artifacts. Furthermore, acetone precipitation resulted in a chromatographic shift. From Figure 2, protein with increased

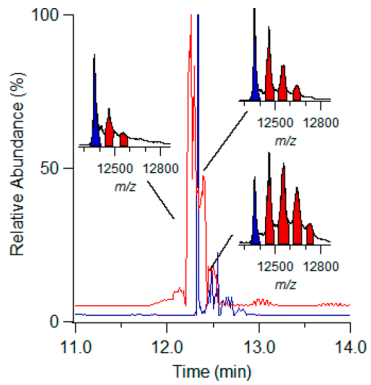

Figure 2. LC-MS analysis of cytochrome c (blue trace) or following acetone precipitation (−20 °C, overnight) (red trace). The insets show extracted MS spectra obtained at varying time points (early, middle, late) along the chromatographic elution profile.

modification is seen to elute later in the chromatogram. Distinctly resolved chromatographic peaks could not be isolated, though MS spectra extracted from the later portion of the eluting peak show a distinctly higher degree of modification. The net effect of this artifact of acetone precipitation is a broadening of the chromatographic profile for the protein. Given the existing challenges of intact protein fractionation with reversed phase chromatography, further reduction of chromatographic performance is a significant concern. Beyond the practical implications, this shift in chromatographic retention also serves to demonstrate that the 98 u artifacts exist in solution, extending beyond the formation of gas phase adducts of neutral or ionic species.

Suspecting a reaction between the protein and acetone, we next explored variables which would likely influence the rate of reaction, including the incubation temperature and time, as well as the pH of the solution. Figures 3 and 4 summarize the results of experiments regarding the effect of time and temperature, respectively. For Figure 3, cytochrome c was incubated in acetone at reduced temperature (−20 °C) over a range of incubation times, with evaporation of the solvent via vacuum concentrator following this incubation period. It should first be noted that the continued exposure of protein to acetone which occurs during solvent evaporation is not reflected in the reported incubation times. Thus, a "time zero" sample is still exposed to acetone (for approximately 30 min in the vacuum concentrator). As seen in Figure 3, a noticeable +98 u signal is readily observed at time zero, accounting for 12% of the total protein signal. The level of modification attributed to the drying phase is minor in comparison to that induced following overnight incubation of the protein in acetone. In this case, over 90% of the MS signal is attributed to +98 u artifacts. Figure 3B quantifies the degree of protein modification as a function of time, as measured through the relative intensity of the unmodified protein signal with respect to the total cytochrome c peak (sum of modified + unmodified peak areas). This reaction does not proceed at constant rate with respect to protein concentration. Modification proceeds more quickly over the first 2 h of reaction (calculated pseudo-first-order rate constant of ∼0.1 h^{-1}). The rate slows considerably, to ∼0.01 h^{-1} (∼10-fold difference), from 2 to 16 h. Considering the impact of precipitation, it is likely that protein aggregation impacts its availability to react. Also of note is the change in higher order protein structure on exposure to acetone. Finally, given that multiple sites of a given protein become modified (3 distinct artifact peaks are seen in Figure 3A), one expects certain sites to be more reactive than others.

The impact of incubation temperature for precipitation in acetone is highlighted in Figure 4. In these experiments, incubation of cytochrome c in acetone was performed for a constant 1 h period, though at varying temperatures. It is immediately apparent that exposure to acetone at higher temperature will dramatically increase the level of protein modification. While over 70% of the protein signal is observed in unmodified form following incubated at −20 °C, the level reduced to only 10% unmodified protein once the temperature is raised to 0 °C. Higher temperatures also cause a significant increase in intensity for the multiply modified protein (Figure 4B), with up to five +98 u artifacts observed in the MS spectrum (incubation at 20 °C). It is feasible that far more reactive sites are involved in the modification; the signals may constitute a distribution of reaction sites and may also incorporate more than one type of protein chemical moiety.

Acetone precipitation is routinely performed across multiple laboratories, though conditions for precipitation are not standardized. Typically, samples are precipitated at reduced temperatures (−20 to 4 °C), though room temperature precipitation is also possible. Incubation periods also vary considerably (1 h or overnight incubations are typical). We have shown that longer incubation at higher temperature lead

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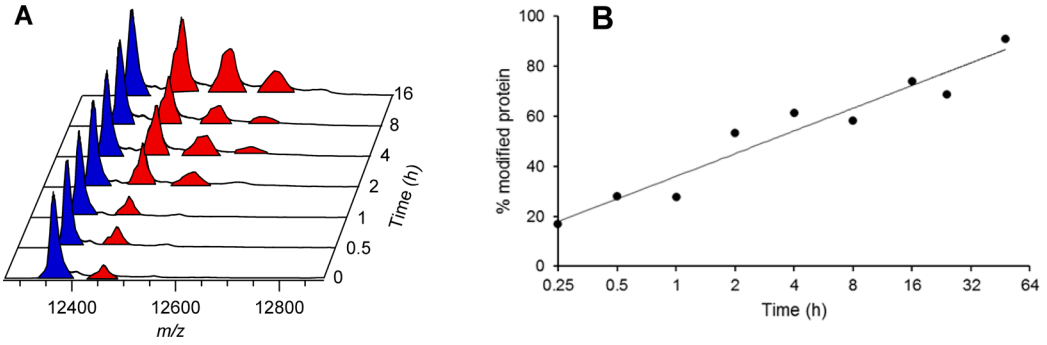

Figure 3. (A) Deconvoluted MS spectra of cytochrome c following incubation in 80% acetone (−20 °C) for defined incubation periods, prior to solvent evaporation in a vacuum concentrator. (B) The level of protein modification increases with longer incubation times.

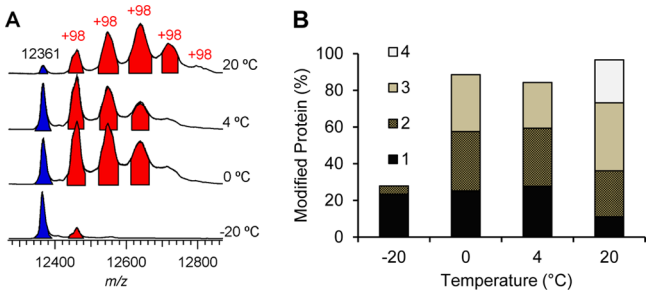

Figure 4. (A) Influence of temperature on the formation of +98 u peaks. Cytochrome c was incubated in 80% acetone for 1 h at the temperatures specified in the plot, prior to solvent evaporation in a vacuum concentrator. (B) The level of modification is quantified as a function of temperature, noting peak areas for protein with 1−4 modifications.

to a significantly greater rate of modification. One must also realize that in conventional precipitation, the protein is also exposed to acetone during centrifugation to isolate the protein pellet. The centrifugation step may take place at reduced temperature, though rarely as low as −20 °C. Following decanting of the solvent, the presence of residual supernatant is unavoidable, and the final drying stage will also expose the protein to acetone, for varying times and temperatures. Certain laboratories allow the protein pellet to stand at room temperature for final drying; others may heat the sample to enhance drying. These practices will impact the level of modification anticipated upon incubation in acetone. To standardize this variability, the results reported here employed a seemingly unusual practice of evaporating the entire solvent via vacuum concentrator. Obviously, such a practice negates the purpose of acetone precipitation, but it was necessary as a controlled method of assessing incubation temperature and time. To confirm that the +98 u peaks are not simply an artifact of the solvent evaporation stage, we also performed conventional acetone precipitation (1 h, −20 °C), wherein the bulk of the acetone is removed via pipetting of the supernatant following centrifugation (residual acetone evaporated in fume hood at room temperature). MS spectra are presented as Figure S3. Compared to the solvent drying protocol, conventional precipitation resulted in a higher degree of protein modification. This was to be expected, as the higher level of modification can be attributed to exposure of protein to acetone at a relatively high temperature (20 °C) during the final drying of the residual solvent.

Based on these results, while it could be recommended to maintain reduced temperatures and brief incubation periods, we note that conventional acetone precipitation still presents the potential for a significant level of protein modification, as observed through +98 u satellite peaks on cytochrome c. Without further reducing the level of protein modification, acetone would be viewed as an undesirable solvent choice for proteomics workflows.

The pH of a solution is an important variable which impacts the recovery of proteins through solvent precipitation. Generally, solutions approaching the isoelectric point of a protein will maximize yield.[37] Adjusting the solution pH may not be practical (if the p*I* is unknown) or even possible when a complex proteomic mixture is precipitated (no single pH will match the p*I* for all proteins). For this reason, it is not a common practice to adjust pH when performing organic solvent. Perhaps as an exception to this, we note the widespread use of trichloroacetic acid (TCA), in conjunction with acetone for protein precipitation.[38] While proteins may be precipitated through exclusive addition of TCA, the acid is frequently paired with acetone (e.g., 10% TCA in 90% acetone) or, alternatively, using acetone as a wash solvent following TCA precipitation.

As shown in Figure 5, the pH of the initial solution has a controlling influence on the reaction. Formation of +98 u

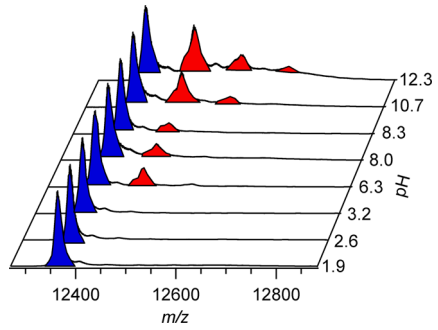

Figure 5. Deconvoluted MS spectra of cytochrome c prepared in solutions of varying pH and precipitated in 80% acetone (−20 °C, 1 h). Note the shoulder on the red peak, which corresponds to the partially resolved series of ions with masses +84 and +98 u.

artifacts is suppressed when acetone is added to acidified cytochrome c solutions near or below pH 3. This is true regardless of the acid used. The MS spectra of Figure 5 include solutions of cytochrome c prepared in 0.1% trifluoroacetic acid (pH 1.9), 0.1% formic acid (pH 2.6), and 0.1% acetic acid (pH 3.2). Lowering the pH with HCl also suppresses the formation of 98 u artifacts (not shown). By contrast, incubation in acetone at neutral or basic pH results in an increasing level of

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protein modification. A typical buffering solution (Tris pH 8 or ammonium bicarbonate pH 8.3) shows minimal difference in the level of modification as compared to that of a purely aqueous solution. However, adjusting to higher pH with ammonium hydroxide further increased the level of modification. Based on these findings, it can be concluded that protein precipitation is best conducted on solutions adjusted to acidic pH prior to addition of the acetone. The use of TCA/acetone precipitation for example would meet these conditions, as would other types of acid, thereby minimizing the risk of inadvertent protein modification and permitting the use of acetone in a proteomics workflow.

Still concerning the pH of the solution, we attempted to reverse the modification by incubating an acetone-precipitated protein in acidic solution following removal of the solvent. It is noted that all MS spectra reported here incorporated an acidic solution (0.1% formic acid) to resolubilize the pellet, with further use of this solvent as mobile phase for LC-ESI-MS. We also incubated the protein pellet in 80% concentrated formic acid, though in all cases the +98 u artifacts remain present. The final reaction product is therefore concluded to be stable in acidic environments, despite the controlling influence of solution pH on the initial reaction.

For ease of comparison, the data presented thus far constitute modification of cytochrome c. Satellite peaks are observed on other proteins and to highly varying degrees depending on the protein. Figure 6 displays MS spectra

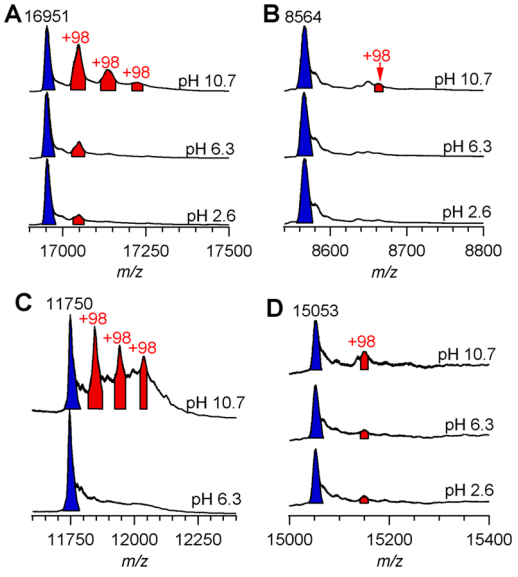

Figure 6. Deconvoluted mass spectra of acetone-precipitated proteins prepared from acidic, neutral and basic solutions: (A) myoglobin, (B) ubiquitin, (C) apo-cytochrome c, and (D) hemoglobin (alpha chain shown).

following acetone precipitation of standard proteins including myoglobin, ubiquitin, hemoglobin, as well as apo-cytochrome c (i.e., in the absence of a heme moiety). Proteins were precipitated in acidic, neutral, and basic solutions to confirm the impact of solution pH on the reaction with acetone. We note the highest degree of modification occurred on heme-containing proteins (cytochrome c, myoglobin, hemoglobin). Suspecting an involvement of the heme group to either catalyze the reaction, or as the chemical moiety involved in the modification, we prepared a sample of apo-cytochrome c, thereby removing the heme group from the system. As seen in Figure 6, a significant level of modification is present on apo-cytochrome c following incubation in acetone, indicating that the amino acid side chains or peptide backbone of the protein is directly modified in acetone. The presence of the heme group may accelerate the reaction, though it does not appear to be required.

While it is concluded that +98 u artifacts can occur on multiple proteins exposed to acetone, we also note this reaction does not occur for all polypeptides. In particular, we examined several small, synthetic peptides in an attempt to localize the site of modification. These peptides were designed to include amine residues (lysine, histidine, arginine, N-terminus), as these were suspected as likely sites for modification in acetone. The resulting MS spectra are provided in Figure S4. Interestingly, while none of the smaller polypeptide segments generated +98 u artifacts, we did observe other mass shifts following incubation in acetone. More specifically, a +112 u artifact was observed on all peptides examined. The exact mass of this artifact was determined as 112.052 u (see Figure S5 for a sample high-resolution MS spectrum), suggesting $C_6H_8O_2$ (4 ppm mass error to observed mass) as the most probable formula. The relative intensity of the +112 u artifact varied for different peptides. MS/MS analysis of the satellite ion confirms the artifact to be a chemical modification of the peptide. For a peptide with amino acid sequence PFHL, the modification was localized to the histidine residue (Figure S6). A mechanistic explanation for the origin of this +112 u modification remains to be determined. However, these artifacts highlight the chemical complexity of acetone and point to a multitude of possible reactions with polypeptide segments. Nonetheless, an absence of +98 u artifacts generated at the peptide level lends a theory that this specific modification requires intact protein to catalyze the reaction. Larger proteins can adopt a complex tertiary structure, offering potential for multiple sites to catalyze a specific reaction. Hydrophobic pockets of the protein may sequester reaction substrates or stabilize intermediates at local sites within the three-dimensional structure of the protein. These local environments of the protein therefore possess a range of chemical properties distinct from the bulk solvent, which may be necessary to drive the reaction forward.

Given the apparent stability of the +98 u modification in both acidic and basic environments, we attempted to localize the reaction site(s) on the protein through bottom-up LC-MS/MS analysis of a tryptic digest following acetone incubation of the intact protein. We employed BSA as a test protein, together with a water-solubilized *E. coli* protein extraction. Searching for +98.0732 u modification without restriction to any specific amino acid, we note the product to be localized to amine-containing amino acids (K, H, and possibly R). While bottom-up LC/MS/MS analysis identified nearly 1500 unique peptides, only 17 were observed as being tentatively modification by acetone (see Table S1). Of these, 7 were assigned to histidine, while 10 were assigned to lysine residues This is a rather low rate of modification and may be a consequence of the level of modification being protein or sequence specific or possibly that the modification is unstable during the digestion process or through MS/MS analysis. Nonetheless, sample MS/MS spectra provided in Figure S7 show localization of the modification to a histidine residue of the BSA peptide hLVDEPQNLIK, as well as a lysine residue on the tryptic peptide SLDDFLIkQ isolated from the *E. coli* DNA-binding protein H-NS (uniprot accession no. P0ACF8). We note that acetone was fully removed from

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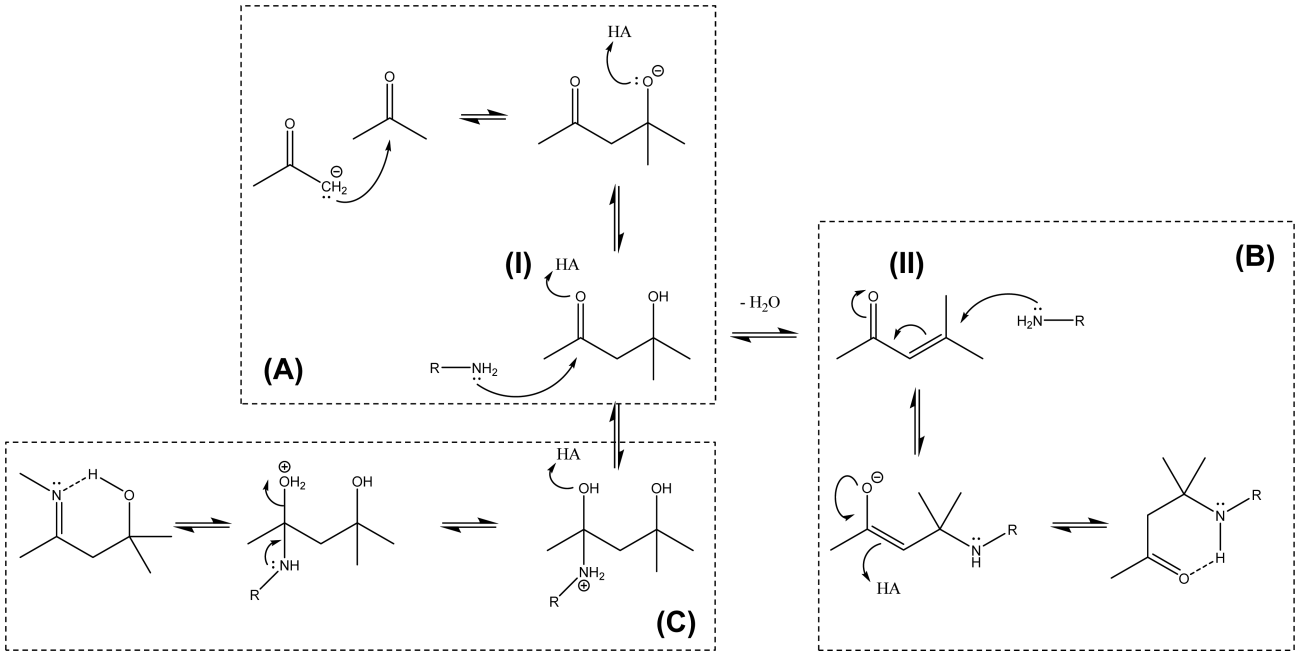
Scheme 1. Proposed Reactions of Diacetone Alcohol (I) or Mesityl Oxide (II) with Protein



the system prior to tryptic digestion; thus, the N-terminus of the resulting peptides would not be available for reaction. These results do not preclude the possibility of modification at other protein residues (e.g., cysteine).

Considering the chemical origin of the +98 u artifact, the mass shift implies an involvement of two molecules of acetone, coupled with the loss of water ($2 \times 58 - 18 = 98$ u). The exact mass we observed (98.08 ± 0.01 u) suggests addition of the chemical moiety $C_6H_{10}O$ (error to observed mass = 69 ppm), which is consistent with the proposed reaction. The only other potential chemical adduct within a 100 ppm tolerance would be $C_5H_{10}N_2$, though such a modification cannot be explained through a reaction with acetone. While the addition may occur through direct reaction with acetone in two discrete steps, an alternative hypothesis would be a single step addition onto the protein. Self-aldol condensation of acetone to form diacetone alcohol (**I**), 116 u, can be catalyzed under acidic or basic conditions (Box A of Scheme 1), which subsequently dehydrates to form mesityl oxide (**II**), 98 u. This reaction is also known to be catalyzed over ion-exchange resin.[39] Given the acid/base properties and localized charges of a protein, the inclusion of a protein may accelerate the aldol addition reaction. Interestingly, the presence of Fe^{3+} has also been shown to favor the formation of diacetone alcohol.[40] This would be consistent with the high level of modification we observe on heme-containing proteins. To test the hypothesis of a single-step addition, we incubated cytochrome c in solutions of 0.1% diacetone alcohol or 0.1% mesityl oxide, prepared in acetonitrile. As seen in Figure 7, reactions with each of these compounds resulted in the formation of a +98 u artifact. Consistent with a modification observed at the peptide level, incubation with mesityl oxide also reveals a prominent +112 u product. Proposed reactions between diacetone alcohol or mesityl oxide and protein are summarized in Scheme 1. Noting the stability of the product in acidic environments, the conjugate addition of mesityl oxide (Box B of Scheme 1) is viewed as a more favorable product. Formation of the imine through condensation with diacetone alcohol (Box C of

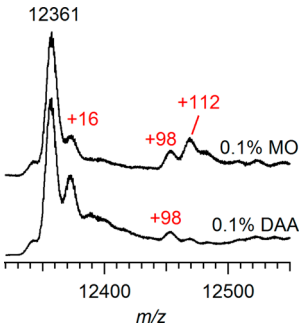


Figure 7. Cytochrome c incubated in acetonitrile with 0.1% MO or 0.1% DAA (−20 °C, 1 h).

Scheme 1) would be a reversible process and therefore requires removal of water to stabilize the product. This may be possible given the higher order structure of the protein. One also cannot preclude the dehydration of diacetone to mesityl oxide prior to addition onto the protein. Reactions at other nucleophilic centers of the protein (e.g., cysteine) may also be possible, though to date, such products have yet to be directly observed by MS/MS.

■ CONCLUSIONS

Herein, we identified a novel chemical modification of occurring on intact proteins following incubation in acetone. The modification appears stable in acidic conditions, though formation of the artifact is strongly dependent on solution pH. The modification is suppressed when proteins are incubated with acetone in acidic solutions. The modification site was localized to histidine, lysine, and arginine residues, though other reaction sites on the protein may exist. The product is speculated to arise following formation of mesityl oxide via aldol condensation of acetone. Given the widespread prevalence of acetone precipitation in proteomics workflows, researchers are cautioned on the use of this solvent, with a recommendation to acidify the protein solution prior to

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addition of acetone. Inclusion of a dynamic modification, +98.0732 u ($C_6H_{10}O$), at lysine, arginine, and histidine residues during MS/MS spectral database searching may account for the presence of this novel modification. However, it is apparent that the chemistry of proteins and peptides in acetone is rich in complexity, with multiple modifications being possible.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b00841.

High-resolution MS spectrum of cytochrome c modified by acetone. MS spectra of cytochrome c incubated in methanol and acetonitrile. Comparison of the level of protein modification with conventional acetone precipitation or drying the solvent by vacuum concentrator. MS spectra of synthetic peptides following incubation in acetone. High-resolution MS spectrum of a synthetic peptide following incubation in acetone. MS/MS analysis of a synthetic peptide localizing the +112 u modification. MS/MS of tryptic peptides localizing the +98 u modification to specific amino acid residues. Table listing *E. coli* tryptic peptides identified with a +98.073 Da modification (acetone). (PDF)

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Notes

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■ ABBREVIATIONS

DAA, diacetone alcohol; MO, mesityl oxide

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