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Phosphate Selective Uranyl Photo-Affinity Cleavage of Proteins. Determination of Phosphorylation Sites

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Phosphorylation of proteins is one of the most important mechanisms in cellular signaling and is involved in cellular processes such as metabolism, transcription, translation, cell cycle, movement, apoptosis, and differentiation. Phosphorylation takes place at serine, threonine, and tyrosine residues in a 1000:100:1 ratio, and it has been estimated that 30% of all proteins are reversibly phosphorylated at one or multiple sites at some point during their lifetime.[1] This is facilitated by the high number of kinases and phosphatases, which constitute 2% of all human genome genes.[2–3] Phosphorylation of proteins is a reversible process, and proteins can be phosphorylated at substoichiometric levels. For proteins containing multiple phosphorylation sites, each site can be associated with a different function; this makes the particular function in question dependent on the phosphorylation pattern.

To understand the role of dynamic protein phosphorylation, the identification of the exact sites and extent of phosphorylation is crucial. At present, state-of-the-art techniques for investigation of protein phosphorylation make use of mass spectrometry (MS), subsequent to protease digests (most often trypsin) of an isolated phosphoprotein or of the entire phosphoproteome in a cell lysate. Secondly, phosphopeptide enrichment is carried out to reduce the number of peptides to be analyzed. Finally N-terminal sequencing and/or MS analysis are performed to identify the specific positions of phosphorylation.[4–6] Although different MS approaches have been used for detecting phosphorylated positions in the proteome, limitations and difficulties remain concerning signal suppression of phosphate containing peptides, dephosphorylation, difficulties in achieving coverage of the full length of long peptides, peptides present in low amounts, peptides phosphorylated at substoichiometric levels, and finally, difficulties in distinguishing among multiple possible phosphorylation sites within in a given peptide fragment. Thus, analysis of peptides after trypsin digests is not straightforward and determination of the exact site of phosphorylation often fails for recovered phosphopeptides.[7–8] Therefore alternative approaches have been considered in order to develop improved methods for phosphorylation site determination in the proteome.

An attractive goal has been to develop phosphospecific proteases in order to reduce the amount of peptide products, which have to be analyzed. Furthermore, such a protease would generate peptide products containing the phosphoamino acid residue positioned either at the N or C terminus thereby significantly simplifying MS analysis and the identification of the phosphorylation sites. Although no phosphospecific protease has so far been found in nature, a chemical approach has been developed where phosphoserines and phosphothreonines are converted into lysine analogues (amino-ethyl cysteine and β-methyl S-ethyl cysteine) to generate cleavage sites for lysine specific proteases.[9–10] However, this methodology is both complex and laborious and involves several chemical and enzymatic modifications, and most importantly, induces problems in distinguishing naturally occurring lysines from those generated from phosphorylated serine and threonine residues.

Uranyl photocleavage has for two decades been used for studying protein–double-stranded (ds) DNA interactions, drug–dsDNA interactions, and the interactions of metal-ions with nucleic acids.[11–16] Uranyl photocleavage of nucleic acids is based on the high affinity of the divalent uranyl cation for phosphates in the backbone of nucleic acids and the strong oxidation power of the excited state of the uranyl ion. Upon excitation of the bound uranyl ion, the high oxidation potential of uranyl induces breakage at the sugar-phosphate backbone at each nucleotide. It was recently reported that proteins can also be photocleaved by uranyl but at low efficiency.[17] In view of the high affinity of the uranyl(VI) ion for phosphate, we have now systematically analyzed whether phosphorylated sites present in proteins could recruit the uranyl ion for subsequent cleavage. We find that both the specificity as well as the efficiency of the photocleavage reaction is increased in the presence of phosphorylated residues and this opens the potential application for detection of phosphorylation sites in proteins by uranyl photocleavage.

To examine whether site-specific cleavage at phoshorylated residues in proteins could be induced by uranyl photocleavage, three different phosphoproteins were selected as model systems: α-casein, β-casein, and ovalbumin. The three proteins differ in size, structure, and extent of phosphorylation, and represent different patterns of phosphorylation in proteins. Initially all three proteins were subjected to uranyl photocleavage at different uranyl/protein ratios and irradiated at 320 nm. The

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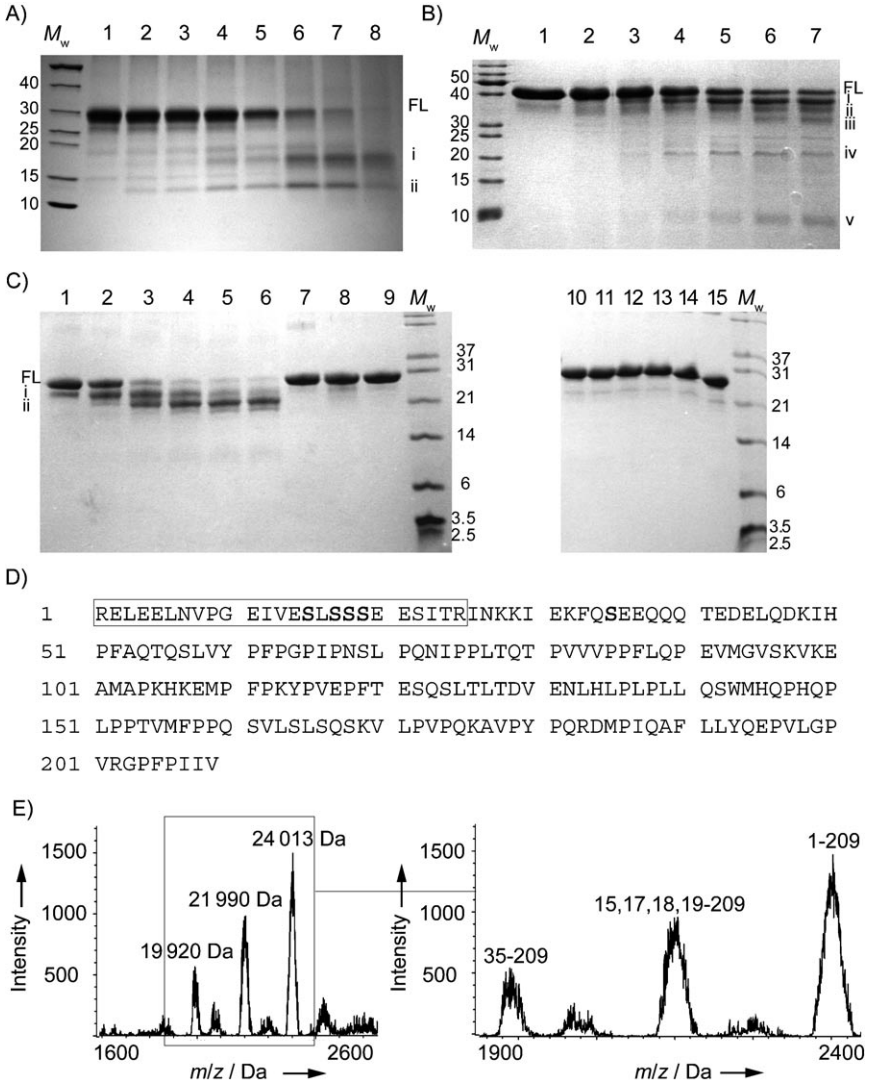


Figure 1. Uranyl photocleavage of α-casein, ovalbumin and β-casein. A) Uranyl photocleavage of α-casein. Aliquots of α-casein were treated with varying amounts of uranyl. In the samples of lanes 2–8 a molar ratio between UO_2^{2+} and protein of: 0.25, 0.5, 1, 2, 4, 8, and 16, respectively, was used. Irradiation was performed at 320 nm for 10 min. The sample of lane 1 was irradiated for 10 min in the absence of UO_2^{2+}, M_W: Molecular weight markers. B) Uranyl photocleavage of ovalbumin. Aliquots of ovalbumin were treated with varying amounts of uranyl. In the samples of lanes 2–7 a molar ratio between UO_2^{2+} and ovalbumin of; 0.5, 1, 2, 4, 8, and 16, respectively was used. Irradiation was performed at 320 nm for 10 min. In lane 1 the sample was irradiated for 10 min in the absence of UO_2^{2+}. M_W: Molecular weight markers. C) Uranyl photocleavage of β-casein. Aliquots of β-casein were treated with varying amounts of uranyl and irradiated for 10 min at 320 nm. Lanes 1–6: The molar ratio of UO_2^{2+} to β-casein was: 0.5, 1, 2, 3, 4, and 5, respectively. Lanes 10–12 the molar ratio of UO_2^{2+} and dephosphorylated β-casein was: 1, 3, and 5 respectively. The sample of lanes 7 and 13 was irradiated in the absence of UO_2^{2+}, the sample of lanes 8 and 14 contained a molar ratio of UO_2^{2+} and β-casein of 5 with no irradiation. Lanes 9 and 15: sample control of stock solutions. M_W: Molecular weight markers. D) Sequence of β-casein. Phosphorylated residues are marked as bold S. The tryptic peptide, β-cas 1–25, is boxed in the sequence E) MS-ESI analysis of uranyl photocleaved β-casein. Aliquots of β-casein was treated with UO_2^{2+} at a UO_2^{2+} to β-casein ratio of three and irradiated for 10 min at 320 nm. The sample was analyzed by LC-ESI-MS. Prior to analysis a desalting step was introduced, thereby removing all peptides with a molecular mass below 10 kDa. Intensity was measured in arbitrary units.

cleavage products were analyzed by SDS-PAGE as shown in Figure 1 A–C.

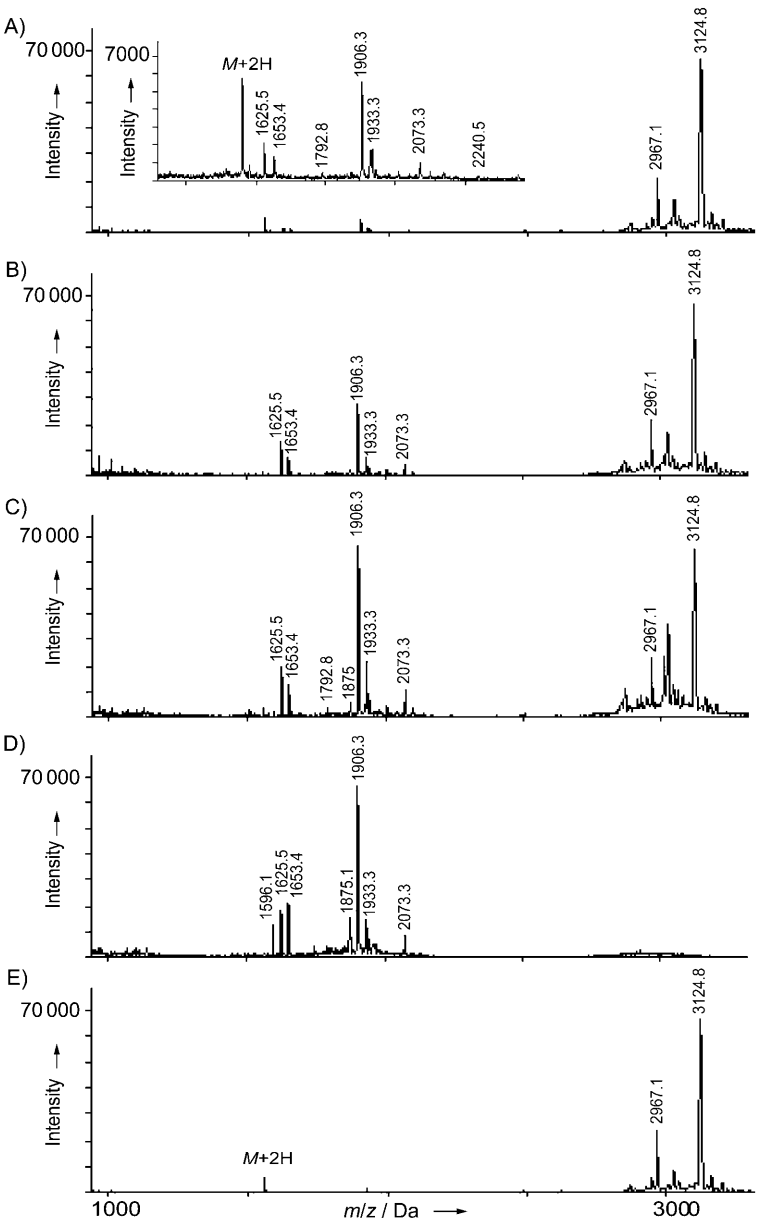
Bovine α-casein contains serine phosphorylation sites at residue 56, 61, 63, 79, 81, 82, 83, 90, and 130.[18] The phosphorylated α-casein was cleaved almost completely by uranyl photo treatment and two major products, a broad band with migration between 15 and 20 kDa, as well as a sharper band with apparent mass around 12 kDa were detected by SDS-PAGE (Figure 1 A, i and ii). If cleavage occurs N-proximal to the phosphorylated residues, C-terminal fragments with masses of 19.0, 18.4, 18.2 (cleavage N-proximal to position 56, 61, and 63), 16.2, 15.9, 15.8, 15.6, 14.7 (cleavage N-proximal to the cluster of position 79, 81, 83, and 90), and 9.9 kDa (cleavage N-proximal to position 130), respectively, would appear. Accordingly, the broad band between 15 and 20 might reflect cleavage at some or all of the phosphorylated residues. Notably, uranyl photocleavage of the nonphosphorylated form of α-casein was not observed under these conditions (data not shown). Analogously, uranyl photocleavage of chicken ovalbumin also gave rise to distinct cleavage products as analyzed by SDS PAGE (Figure 1 B). Chicken ovalbumin contains two well separated phosphorylated serine residues at position 69 and 345, respectively.[19] MS and N-terminal sequencing of the small cleavage product below 10 kDa, (Figure 1 B v) showed that cleavage had occurred N-proximal at residues 66, 68, 69, 345, 346, 347, 348, and 349, showing that uranyl photocleavage indeed occurred at the phosphorylated sites at *p*-Ser 69 and *p*-Ser345 (data not shown).

Bovine β-casein contains 209 amino acid residues including four phosphoserines clustered at residue 15, 17, 18, and 19 (p-Ser15, 17–19) and a single phosphorylated serine at residue 35 (p-Ser35) (Figure 1 D).[20] As shown by the data represented in Figure 1 C, phosphorylated but not the nonphosphorylated β-casein was efficiently and selectively photocleaved by uranyl. Irradiation at 320 nm in the presence of uranyl resulted in conversion of the phosphorylated protein into two well-defined cleavage products as analyzed by SDS-PAGE (Figure 1 C, lanes 1–6). The efficiency and specificity of the photocleavage were

clearly uranyl dose-dependent. At low uranyl/protein ratios one cleavage product was observed, and above a uranyl/protein ratio of two the second cleavage product was the more abundant (Figure 1 C i and ii). At high uranyl/protein ratios a third low-intensity band was also visible. The band representing full-length β-casein was virtually absent at a uranyl/protein ratio of five demonstrating very high efficiency of the cleavage. In contrast, no cleavage of phosphorylated β-casein was observed upon irradiation in the absence of uranyl or when incubated with uranyl without irradiation (Figure 1 C, lanes 7 and 8). Even more importantly, no cleavage was observed in a similar experiment using enzymatically dephosphorylated β-casein even at a high uranyl/protein ratio of 5 (Figure 1 C, lanes 10–15). The specificity of cleavage was further examined in a mixture of phosphorylated β-casein and bovine serum albumin (BSA; Supporting Information). In this case phosphorylated β-casein was preferentially cleaved over BSA.

As the uranyl photocleavage of purified β-casein yielded the most unambiguous results, we chose to analyze the fate of this protein in more detail. In order to characterize the observed cleavage products and to identify the cleavage positions in the phosphoprotein, a sample of uranyl photo cleaved β-casein was analyzed by LC-ESI-MS (Figure 1 E). Three intense, broad peaks with masses of 24013 Da (full-length protein, 1–209, expected mass 24023 Da), 21992 Da (residue 15–17, 19–209, expected masses 22415, 22135, 21888, and 21721 Da) and 19920 Da (residue 35–209, expected mass 19,789 Da) were apparent. The masses agree nicely with those expected from cleavage sites proximal to the phosphorylated residues.

To accurately determine the exact cleavage sites, N-terminal sequencing of the cleavage products was performed. The smaller product gave the sequence SEEQQQTED, indicating that cleavage had indeed occurred at the N-terminal side juxtaposed *p*-Ser35. However, with this method we did not succeed in determining the precise cleavage sites responsible for the larger SDS-PAGE cleavage product from the *p*-Ser15, 17–19 region. Therefore, we decided to perform an analogous uranyl photocleavage experiment using a tryptic peptide fragment from β-casein (β-cas 1–25) containing the phosphoserine cluster (Figure 1 D) at *p*-Ser15, 17–19. Fragments of masses of 1625, 1906, 2073, and 2240 Da would be expected if cleavage occurred N-proximal to each of the phosphoserines. The time course of the cleavage reaction showed that two fragments of masses 1625.5 and 1906.3 Da were formed upon irradiation indicating specific cleavage N-proximal to *p*-Ser15 and to *p*-Ser17 (Figure 2). Furthermore, a peak at 2073.3 Da could be clearly identified after 10 min of irradiation



F)

Fragment	Expected mass	Measured mass ($+H^+$)
1-25	3123.35	3124.8
1-18	2240.42	Not obsered
1-17	2073.33	2073.3
1-16	1906.24	1906.3
1-14	1625.98	1625.5

Figure 2. Uranyl photocleavage of β-casein peptide 1–25. All samples contained peptide and UO_2^{2+} in a molar ratio of 1:0.5 (except the sample of panel E, which contained no UO_2^{2+}). Irradiation time was 1 min in panel A), and 3, 10, 20, and 20 min in panel B), C), D), and E), respectively. The masses of fragments 1–14, 16, and 17 were determined to be 1625.5, 1906.3, and 2073.3 Da, respectively, corresponding to N-terminal fragments from uranyl photocleavage N-terminal to pSer 15, 17, and 18, respectively. The peptide starting material (panel E) was a mixture of β-casein 1–25 and 2–25 with masses of 3124.8 and 2967.1 Da resulting from incomplete trypsin digestion a R1. The signal labeled $[M+2H]^+$ in panel E is the doubly charged β-casein 1–25. Intensity was measured in arbitrary units. F) Expected and correspondingly detected masses of the uranyl/UVA-cleaved peptide fragments are listed.

consistent with cleavage N-proximal to *p*-Ser18. In contrast the mass corresponding to cleavage N-proximal to *p*-Ser19 was not observed. The expected and detected masses of the peptides are summarized in Figure 2F. It is evident that the two analyses performed on β-casein showed cleavage exclusively in the *p*-Ser15, 17–19 regions (upper band in Figure 1C) at low uranyl/protein ratio and when using an elevated uranyl/protein ratio, cleavage N-proximal to *p*-Ser35 took place as well. It is worth noting that uranyl photocleavage is apparently able to resolve phosphorylation even at individual and closely positioned sites that might be adjacent.

Experimental data support the conclusion that uranyl induced photocleavage of nucleic acids is caused by photo-oxidation of the riboses adjacent to the phosphate to which uranyl is complexed, and the reaction has been proposed to involve direct hydrogen abstraction by the excited uranyl ion.[21] Most surprisingly, the presented MS and N-terminal sequencing data suggest that uranyl photocleavage of proteins is primarily occurring via photoinduced hydrolysis of the amide bond, producing peptide products with free amino and carboxy termini, analogous to protein cleavage by $Fe^{II/III}$ complexes.[22] The presence of cleavage sites resulting from uranyl photocleavage at sites adjacent to the phosphorylated residues in ovalbumin indicates that some cleavage related to structural motifs might also be present. This is supported by the lack of cleavage sites adjacent to phosphoserines in β-casein, whose structure, contrary to the compact tertiary structure of ovalbumin, is looser and has been described as rheomorphic. However, the exact mechanism of the reaction for uranyl photocleavage warrants further study.

The dramatic quantitative and qualitative differences in uranyl photocleavage between the phosphorylated and non-phosphorylated β-casein (and α-casein) clearly demonstrate that at stoichiometric amounts of uranyl to protein any background cleavage (from the low-affinity site) in the protein was very inefficient compared to phosphorylation directed cleavage. This clearly identifies uranyl as a phosphoamino acid selective photocleavage reagent. Although the full potential of the uranyl photocleavage strategy cannot yet be assessed, we suggest that the technique will offer significant advantages in determination of protein phosphorylation sites with high precision, especially when combined with automated MS techniques.

The present results do not address whether uranyl photocleavage at phosphothreonine and phosphotyrosine takes place with the same efficiency and specificity. However, 90% of all proteomic phosphorylation sites involve serine and most likely analogous results will be obtained for phosphothreonine (and possibly phosphotyrosine) containing proteins, as the affinity is related to the phosphogroup and not the specific residue type.

A detailed understanding of the uranyl-protein photochemistry as well as of the influence of sequence context on cleavage efficiency is warranted in further studies. Furthermore, uranyl photocleavage should have interesting applications in protein engineering, using phosphoserine peptide tags that subsequently can be removed by uranyl directed photocleavage. Finally, the finding that phosphorylated proteins are specifically cleaved by uranyl might be relevant for the evaluation of uranyl (photo) toxicity because phosphorylated proteins are central regulators of intracellular communication.

Experimental Section

Purification of β-casein: The purification protocol was modified from that previously described.[23] Bovine β-casein was purified from consume skimmed milk (100 mL). Whole casein was precipitated at 20 °C with slow addition of HCl (0.2 M) until pH reached 4.6. The precipitate was collected by centrifugation at 3500 rpm for 1 min and the supernatant discarded. The precipitate was washed with deionized water (50 mL), recollected, redissolved in buffer [20 mL Tris-HCl (20 mM, pH 7.2), urea (4.5 M), DTT (1 mM)] and filtered through a 22 μm sterile filter. Aliquots of whole casein (3 mg) were applied to a MonoQ 5/5 HR column on an ÄKTA FPLC purification system (Amersham pharmacia biotech) and a linear salt gradient was run with buffer A [Tris-HCl (20 mM, pH 7.2), urea (4.5 M), DTT (1 mM)] and buffer B [Tris-HCl (20 mM, pH 7.2), urea (4.5 M), DTT (1 mM), NaCl (0.35 M)]. Fractions containing β-casein eluted around 76.5% B, and were pooled and dialyzed extensively against Tris-HCl (20 mM, pH 7.2), at 4 °C to remove urea. Finally β-casein was concentrated on a Millipore spin filter with a cut off at 10000 Da. The protein was heated to 78 °C for 15 min to prevent autoproteolysis. The final concentration was determined by A_{280} using an absorptivity of 0.46 $cm^2 mg^{-1}$.[24] The batch was diluted to an exact concentration of 1 $mg mL^{-1}$, and separated into aliquots (120 μL) and stored at −20 °C. The phosphorylated peptide; β-cas 1–25, and bovine α-casein were purchased from Sigma. Chicken ovalbumin was purchased from Worthington. All purchased proteins were used without further purification.

Dephosphorylation of β-casein: An aliquot of β-casein (120 μg) was dephosphorylated using CIP (calf intestinal alkaline phosphatase; M0290L, New England BioLabs) (5 units) according to the manufacturer's instructions. To remove all traces of EDTA, which would otherwise complex the reactive uranyl ion, the dephoshorylated protein was extensively dialyzed against Tris-HCl (20 mM, pH 7.2). Finally the protein was concentrated to 1 $mg mL^{-1}$. The efficiency of the dephosphorylation was judged from SDS-PAGE, as the dephosphorylation causes a gel shift of approximately 3 mm on a 15% SDS tris-glycin gel (results not shown). Dephosphorylated α-casein was purchased from Sigma and used without further purification.

Uranyl photocleavage: $UO_2(NO_3)_2$ (CAS: 36478-76-9, Bie & Berntsen) was dissolved in deionized water to a concentration of 100 mM (stable at RT). Prior to reaction, the uranyl solution was diluted 200- or 500-fold with Tris-HCl (20 mM, pH 7.2). The reaction was performed in low binding Eppendorf tubes with each reaction mixture containing protein/peptide (200–350 pmol), NP-40 (0.05%) and uranyl in a molar ratio as indicated in the figure legends. Tris-HCl (20 mM, pH 7.2) was added to a final reaction volume of 20 μL. The reaction was incubated for 15 min on ice. The reaction mixture was transferred to the lids of Eppendorf tubes and placed directly under a Philips TL 40 W/03 fluorescent light tube with $\lambda = 320$ nm and irradiated for 1–20 min at 0 °C as indicated in the figure legends. After irradiation the samples were analyzed by SDS-PAGE, MS and/or N-terminal sequencing. Samples that were not immediately analyzed were stored at −20 °C.

MALDI-TOF MS: Aliquots of uranyl photocleaved β-cas 1–25 were desalted on a Millipore ZipTip according to the manufacturer's

instructions. The peptide was eluted from the ZipTip in 2 μL acetonitrile (50%, *v/v*)/TFA (0.1%, *v/v*) and mixed 1:1 (*v/v*) with HCCA (10 mg mL^{-1} in acetonitrile (50%, *v/v*)/TFA (0.1%, *v/v*). The peptide/matrix mixture was spotted on a MTP anchor chip™ 600/384 TF target plate. The drops were allowed to air dry, and analyzed on a Bruker autoflex III MALDI-TOF mass spectrometer in linear positive mode. A total of 100 shots were averaged for each spectrum. The spectra were analyzed with MoverZ (Proteometrics, Inc)

LC-ESI-MS: LC-ESI-MS analysis was performed on a Bruker electrospray (ESI) MicroTOF Focus coupled to an Agilent 1100 HPLC system where aliquots of uranyl photocleaved β-casein were desalted on a MassPREP™ On-Line Desalting Cartridge (Waters). ES Tuning mix from Agilent was used for "quasi" internal calibration. Spectra deconvolution was done by the maximum entropy method using Maximum Entropy (MaxEnt) Deconvolution software from Spectrum Square Associates, Inc.

***N*-terminal sequencing**: Prior to sequencing uranyl photocleaved β-casein samples were separated by SDS-PAGE. The samples were loaded on a standard, precast 4–20% SDS polyacrylamide gel (Invitrogen) for electrophoresis and blotted to a PVDF membrane for N-terminal sequencing. Selected protein bands were cut from the membrane and sequenced on a Procise Protein Sequencer from Applied Biosystems according to the manufactures instructions using the standard gas-phase PVDF method.

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