Protein Mass Spectrometry

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Simultaneous Assessment of Kinetic, Site-Specific, and Structural Aspects of Enzymatic Protein Phosphorylation**

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Abstract: *Protein phosphorylation is a widespread process forming the mechanistic basis of cellular signaling. Up to now, different aspects, for example, site-specificity, kinetics, role of co-factors, and structure–function relationships have been typically investigated by multiple techniques that are incompatible with one another. The approach introduced here maximizes the amount of information gained on protein (complex) phosphorylation while minimizing sample handling. Using high-resolution native mass spectrometry on intact protein (assemblies) up to 150 kDa we track the sequential incorporation of phosphate groups and map their localization by peptide LC-MS/MS. On two model systems, the protein kinase G and the interplay between Aurora kinase A and Bora, we demonstrate the simultaneous monitoring of various aspects of the phosphorylation process, namely the effect of different cofactors on PKG autophosphorylation and the interaction of AurA and Bora as both an enzyme–substrate pair and physical binding partners.*

Phosphorylation of proteins, where phosphotransferases (kinases) transfer inorganic phosphate from adenosine-5′-triphosphate (ATP) to substrate amino acids (mainly threonine, serine, and tyrosine), is a universal process in biological systems. It affects the activity, conformation, localization, oligomeric state, and/or binding repertoire of the substrate proteins and forms the basis of ubiquitous cellular signaling events.[1] The global high-throughput analysis of dynamic protein phosphorylation (phosphoproteomics) has become feasible and datasets with over ten thousand phosphorylation sites per experiment have been reported.[2] However, more in-depth, diverse, and multidimensional low-throughput analyses are required to fully understand the functional implications of a protein phosphorylation event. Presently, protein phosphorylation is often investigated at separate levels by using disparate methods that require different sample preparation steps or even specific engineering of the proteins. First, at the amino acid sequence level, phosphosite-specific antibodies[3] or mass spectrometric proteomics approaches can be used to gather site-specific information, for example sequence motifs recognized by a kinase and possible cross-talk between different modified sites.[4] Secondly, kinetic biochemical analyses, e.g. radiometric or fluorescence/luminescence assays, can provide information on the reaction rates of each enzyme–substrate pair, the requirements of co-factors, and the influence of environmental conditions on kinase activation, deactivation, and inhibition.[5] Thirdly, a range of structural effects of the phosphorylation event can be interrogated, for example changes in conformation, oligomeric state, and protein complex formation or dissociation,[6] by using structural biology methods such as nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography, and electron microscopy.[7]

Ideally, a single method would allow the simultaneous analysis of protein phosphorylation at all three levels. Mass spectrometric analysis of proteins and protein complexes under non-denaturing conditions (native mass spectrometry)[8] offers the potential to provide information at both the structural and the kinetic level, as phosphate incorporation can be identified by a characteristic mass shift of nominally 80 Da and the non-denaturing setting enables monitoring of changes within noncovalently bound protein assemblies. However, as phosphate incorporation in proteins and protein complexes only causes a minimal mass shift ($< 0.5\,\%$), the mass resolution needs to be sufficient for the detailed recording of sequential phosphorylation events. In 2012 we introduced an Orbitrap mass analyzer with extended mass range[9] that provides very high mass resolution, making it possible, for example, to resolve complex glycosylation profiles on antibodies.[10] Making use of this novel instrument we designed an approach to maximize the amount of information that can be derived from protein phosphorylation while minimizing sample preparation, consumption, and analysis time. High-resolution mass spectrometric analysis of proteins and protein complexes facilitates baseline resolution of differentially phosphorylated proteoforms, enabling us to count the number of sequentially incorporated phosphates, relatively quantify all occurring phospho-isoforms, and visualize transitions on the level of noncovalent protein

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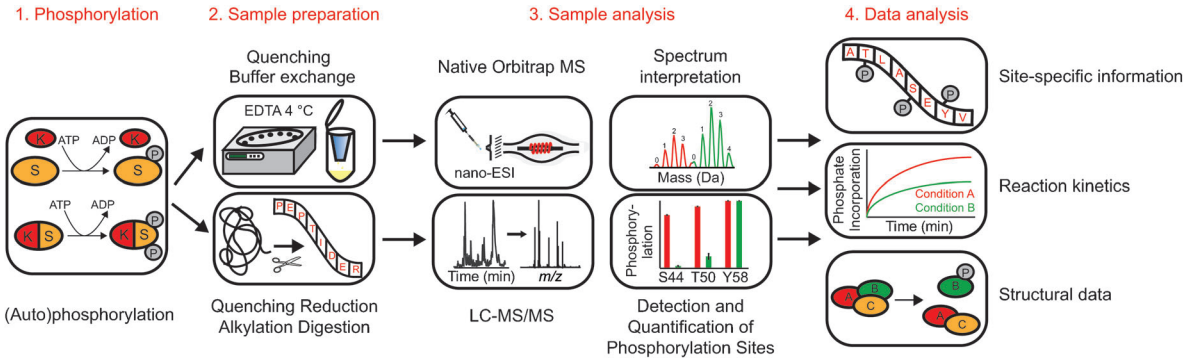

Figure 1. Overview of the comprehensive MS-based method to investigate protein phosphorylation. 1) A kinase (K) and its substrate (S) are incubated with Mg^{2+}, ATP, and other essential cofactors to initiate (auto)phosphorylation. 2,3) Samples are split and prepared for high-resolution native MS analysis on the Orbitrap (top) or bottom-up LC-MS/MS analysis (bottom). 4) Data analysis provides information at the sequence, kinetic, and structural level.

interactions. To obtain information on amino acid sequence level as well, we further extended this method and subjected the phosphorylated proteins, originally generated for the native MS analysis, also to bottom-up liquid-chromatography tandem mass spectrometry (LC-MS/MS), thereby acquiring extensive information on the amino acid sequence and site of the phosphorylation event. The generic workflow outlined in Figure 1 thus provides the opportunity to obtain data on all described facets of protein phosphorylation.

We apply our approach on two challenging and interesting systems: the cGMP-dependent protein kinase G (PKG), which phosphorylates residues from its own N-terminus upon cGMP (cyclic guanosine monophosphate) or cAMP (cyclic adenosine monophosphate) binding-induced activation,[11] and the Aurora kinase A (AurA), which phosphorylates its interaction partner Protein aurora borealis (Bora).[12] PKG is highly expressed in all types of smooth muscle cells, platelets, and certain areas of the brain, playing an important role in synaptic activity and smooth muscle contraction.[13] The interplay of Aurora kinase A and Bora is critical for eukaryotic cells to enter mitosis as they form a protein complex and cooperatively activate polo-like kinase 1.[12] Here, we demonstrate the potential of this integrated mass spectrometric approach to simultaneously characterize site-specific, kinetic, and structural aspects of these enzymatic phosphorylation reactions.

Initially, we probed untreated PKG by native MS (Figure 2a). Based on the mass-to-charge ratios (m/z) of adjacent peaks of the charge state envelope[14] the accurate molecular weight of the kinase is calculated to be 152817.6 Da, which corresponds to the expected mass of homodimeric PKG (Table S1). Figure 2b shows that, as a result of the purification procedure, up to two cAMP molecules are intrinsically attached to PKG and the loading state is increased to four cAMP molecules upon incubation with 30 μM cAMP (species I–V). In the presence of 10 μM cGMP, three to four cGMP molecules are found to bind noncovalently to PKG (species VI–VII), displacing all cAMP molecules, as evidenced by the experimentally determined mass, and reflecting PKG's known higher affinity to cGMP.[15] Owing to the high resolving power of the Orbitrap, we can not only count the number of cyclic nucleotide molecules bound to PKG but also distinguish between the differentially phosphorylated isoforms of each protein/cofactor complex, demonstrating that the predominant proteoform of the PKG dimer has consistently two phosphates incorporated (Figure 2b). To investigate how cyclic nucleotide binding triggers PKG autophosphorylation, we incubated PKG with cAMP or cGMP in the presence of Mg^{2+} and ATP and monitored the reaction over time by native MS. We acquired the PKG spectra with a mass resolution of roughly 6000 (full width at half maximum), facilitating baseline resolution of all different phospho-isoforms. the progressive binding of phosphate is illustrated by a continuous signal shift to higher m/z. We detect up to 13 or 6 phosphorylations on the PKG dimer in the presence of cAMP or cGMP, respectively (Figure 2c). In total, we identify 56 proteoforms of PKG/cyclic nucleotide complexes (Tables S1 and S2). Deconvolution of the mass spectra to the zero charge state allowed us to calculate the weighted average number of phosphate incorporations based on the relative signal intensities (Figure 2d). Evidently, the maximum number of phosphorylations on PKG/cAMP complexes is substantially higher than on PKG/cGMP complexes, as schematically depicted in Figure 2e. This finding is confirmed by quantitative bottom-up LC-MS/MS analysis of the sample representing the final time point (Figure 2f). In line with the native MS data we identify three highly phosphorylated sites per PKG monomer, when PKG interacted with cGMP, but six highly phosphorylated sites for the PKG/cAMP complex, all in the vicinity of the N-terminus. In compliance with the identification of two phosphorylations on purified PKG dimer, residue Thr516 (located on the activation loop) is shown to be fully phosphorylated also without Mg^{2+}/ATP incubation.

Next, we aimed to apply this workflow to investigate the interaction of the kinase domain of Aurora kinase A (32.9 kDa) and an N-terminal fragment of Bora (17.5 kDa). Native MS analysis of AurA and Bora (molar ratio 1:2) confirms the formation of a 1:1 complex after incubation with Mg^{2+} and ATP (Figure 3a), as previously reported by Hutterer et al.[12b] In contrast to the results of co-immunoprecipitation studies with full-length Bora and AurA,[16] we observe complex formation also in the absence of Mg^{2+}/ATP,

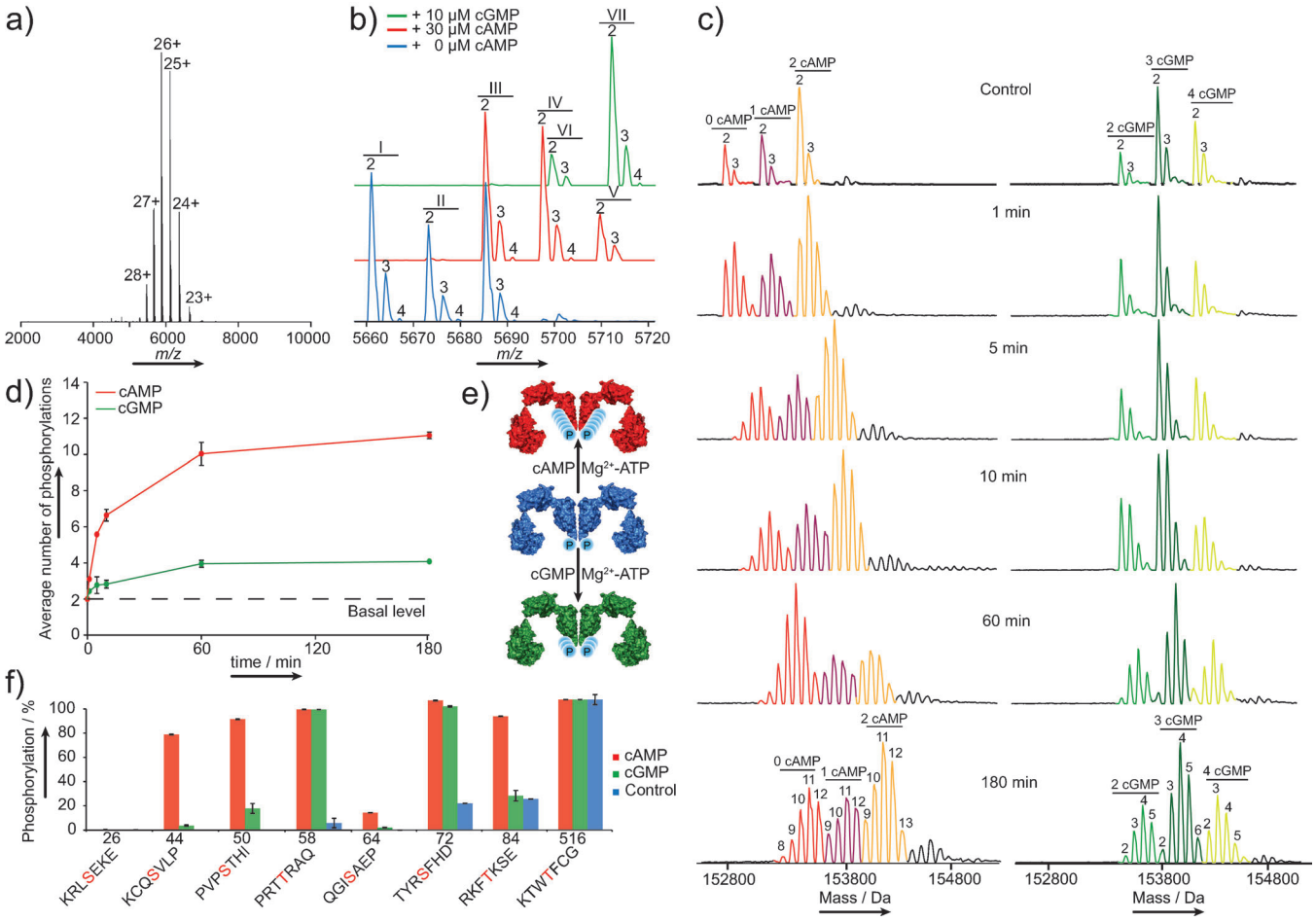

Figure 2. Comprehensive analysis of the cAMP and cGMP mediated autophosphorylation of PKG with native and bottom-up MS. a) Broad-range native mass spectrum of dimeric PKG. The main mass derived from this charge-state envelope is 152817.6 Da. b) Enlargement of the $[M+27\,H]^{27+}$ signal and the observed mass shifts upon addition of cAMP or cGMP. Arabic numerals indicate the number of attached phosphate groups per dimer, roman numerals indicate distinct cyclic nucleotide bound states. c) Native mass spectra illustrating the progressive autophosphorylation of PKG upon incubation with Mg^{2+}/ATP and cAMP or cGMP. d) Changes of the intensity-weighted average number of PKG autophosphorylations over time in the presence of cAMP or cGMP. e) Schematic representation of the differential autophosphorylation of PKG in the presence of cAMP or cGMP. f) Identification and quantification of phosphorylated sites on PKG by bottom-up LC/MS-MS.

that is, with unphosphorylated Bora. Nevertheless, the complex forms more readily following incubation with Mg^{2+}/ATP, indicating a higher AurA binding affinity of phosphorylated Bora. Since we obtained baseline resolution of all phospho-isoforms of AurA, Bora, and the 1:1 complex with our native MS setup we were able to study the enzyme–substrate relationship of AurA and Bora in a time-course experiment. Human AurA, purified from *E. coli* following overexpression, is found to bear up to seven phosphate groups. Quantitative bottom-up LC-MS/MS experiments reveal 100% phosphorylation on Thr288, a modification that is essential for the full enzymatic activity of AurA[17] (Figure S1). Incubating AurA with Mg^{2+} and ATP does not change its phosphorylation state (Figure 3b). AurA is, however, catalytically active as illustrated in Figure 3c. Bora as well as the AurA/Bora complex become increasingly phosphorylated following incubation with Mg^{2+}/ATP. In the control, where no Mg^{2+}/ATP was added, only unphosphorylated Bora is detected and the phospho-isoform distribution of the complex resembles the phosphorylation state of AurA. After 180 min reaction time, up to six phosphorylations are detected on Bora and the complex exhibits a maximum of ten phosphate incorporations. Calculating the weighted average number of phosphorylations confirms that both complex and Bora follow the same trend of progressive phosphorylation towards a saturation point (Figure 3d). The determined average of three phosphorylations on Bora at the last time point is in line with site-specific phosphorylation data derived from quantitative LC-MS/MS experiments where two highly and two moderately phosphorylated sites could be identified (Figure 3e). Of these, Ser59 and Thr144 are likely to be specific AurA targets as their location largely complies with the AurA substrate-recognition sequence.[18] To the best of our knowledge, this is the first report of AurA-specific phosphorylation sites on Bora. The similar phosphorylation time-courses of Bora and the AurA/Bora complex indicate that Bora does not need to form a stable complex with AurA to become phosphorylated. To verify this hypothesis we also studied Bora phosphoryla-

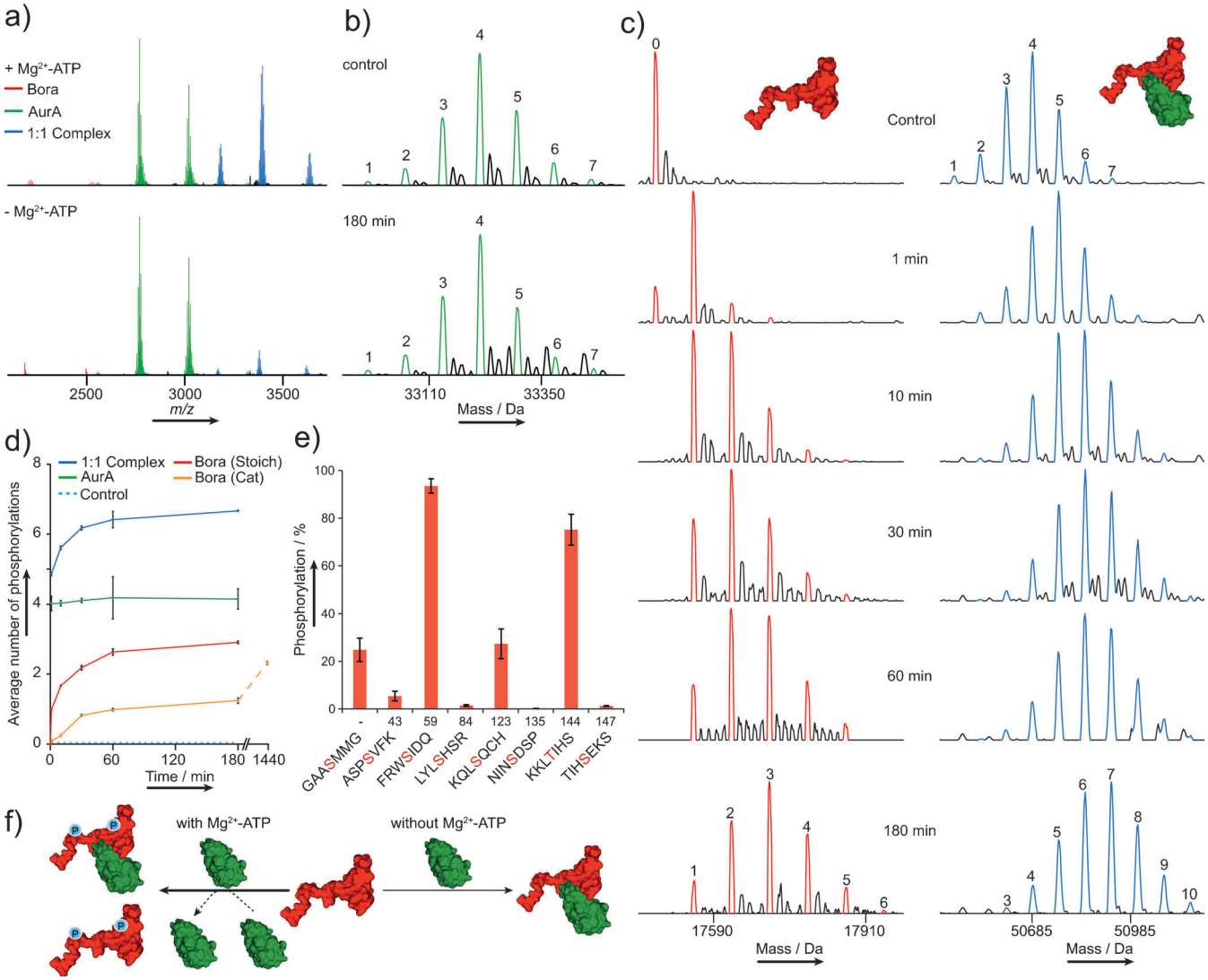

Figure 3. Comprehensive analysis of the interplay between Bora and the kinase Aurora A by native and bottom-up MS. a) Native MS analysis of AurA and Bora after 180 min incubation. The 1:1 complex forms more efficiently with Mg^{2+}/ATP added. b) Purified AurA shows 7 distinct phospho-isoforms before and after incubation with Mg^{2+}/ATP. c) Native MS evidences the progressive phosphorylation of Bora and the AurA/Bora complex in the presence of Mg^{2+}/ATP. d) The weighted average numbers of phosphorylations on Bora and the AurA/Bora complex increase simultaneously over time (AurA/Bora ratio 1:2, "Stoich"). Near-catalytic amounts of AurA facilitate progressive Bora phosphorylation as well ("Cat"). e) Identification and quantification of phosphorylated sites on Bora by bottom-up LC/MS-MS. f) AurA forms a stable complex with unphosphorylated Bora and, conversely, Bora phosphorylation proceeds already at catalytic AurA concentrations. Thus, complex formation and phosphorylation are likely to represent two independent modes of interaction.

tion using near-catalytic amounts of the kinase (AurA/Bora molar ratio 1:100, Figure S2). Although the reaction rate is reduced under these conditions, we still detect progressive Bora phosphorylation up to an average number of approximately 2.5 incorporated phosphates, as expected for a Michaelis–Menten-type enzymatic reaction (Figure 3 d). Bora thus seems to fulfil a dual role as a transiently bound substrate and a stable interaction partner of Aurora kinase A, as previously hypothesized[12b] (Figure 3 f).

In summary, by combining high-resolution native MS and bottom-up LC-MS/MS in one workflow, we are able to characterize enzymatic protein phosphorylation at the level of amino acid sequence modifications, relative reaction kinetics, and structural transitions. Finally, we argue that the strategy presented here can be applied to any enzyme/(protein-)substrate system, providing a generic approach to obtain sequential, kinetic, and structural information in parallel.

Experimental Section

Kinase reactions were carried out at physiological pH in the presence of ATP and magnesium chloride at 30 °C with a 1000-fold molar excess of cAMP or cGMP (PKG) or at room temperature with a twofold excess of Bora (AurA).

Reactions were quenched on ice by addition of EDTA. After buffer exchange to ammonium acetate pH 6.8, samples were analyzed using a modified Orbitrap Exactive Plus mass spectrometer, with settings optimized to obtain high resolution and sensitivity and kept constant for the specific proteins. For LC-MS/MS analysis, reaction

mixtures were denatured, reduced, and alkylated and subsequently digested using trypsin and GluC (PKG) or trypsin (AurA/Bora). After desalting, samples were analyzed on a LTQ-Orbitrap Elite coupled to a Proxeon EASY-nLC 1000. Additional experimental details are available in the Supporting Information.

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