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Identification of peptide binding sites within BSA using rapid, laser-induced covalent cross-linking combined with high-performance mass spectrometry

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Abstract

We are developing a rapid, time-resolved method using laser-activated cross-linking to capture protein-peptide interactions as a means to interrogate the interaction of serum proteins as delivery systems for peptides and other molecules. A model system was established to investigate the interactions between bovine serum albumin (BSA) and two peptides, the tridecapeptide budding-yeast mating pheromone (α -factor) and the decapeptide human gonadotropin-releasing hormone (GnRH). Cross-linking of α -factor, using a biotinylated, photoactivatable *p*-benzoyl-L-phenylalanine (Bpa) modified analog, was energy-dependent and achieved within seconds of laser irradiation. Protein blotting with an avidin probe was used to detect biotinylated species in the BSA-peptide complex. The cross-linked complex was trypsinized and then interrogated with nano-LC-MS/MS to identify the peptide cross-links. Cross-linking was greatly facilitated by Bpa in the peptide, but some cross-linking occurred at higher laser powers and high concentrations of a non-Bpa-modified α -factor. This was supported by experiments using GnRH, a peptide with sequence homology to α -factor, which was likewise found to be cross-linked to BSA by laser irradiation. Analysis of peptides in the mass spectra showed that the binding site for both α -factor and GnRH was in the BSA pocket defined previously as the site for fatty acid binding. This model system validates the use of laser-activation to facilitate cross-linking of Bpa-containing molecules to

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Author contributions: JMB, MH, QC, and RH conceived the work and wrote the manuscript. MH and SK performed the majority of the biological experiments. STK performed the docking experiment. QC and RH performed the Mass-spectrometry analyses. FN designed and synthesized the α -factor peptides used in this study. JMB, MH, RH, QC, STK, and FN contributed to the final manuscript preparation. JMB and RH supervised all aspects of the project.

proteins. The rapid crosslinking procedure and high performance of MS/MS to identify cross-links provides a method to interrogate protein-peptide interactions in a living cell in a time-resolved manner.

While the interactions between proteins and ligands or other proteins in a biological system may occur with high affinity, often these interactions are transient, existing only long enough to transduce a signal or undergo enzymatic modification. Thus, a variety of cross-linking methodologies have been developed to capture these interactions, isolate stable complexes, and then dissect the interactions utilizing enzymatic digestion coupled with tandem mass spectrometry (MS/MS) to identify fragments derived from the peptides in the complex.

Chemical cross-linking agents have been widely used to stabilize protein interactions^{1, 2}. These agents target specific amino acid functional groups to form bonds between residues positioned in close proximity and with the correct geometry. However, chemical cross-linkers are reactive at all times and when studying interactions *in vivo* or in cell lysates, can result in the formation of protein conjugates that can be very challenging to interpret.

In contrast to chemical cross-linking agents, photoactivation can be temporally controlled, and photoreactive cross-linkers can be used to specifically target interactions between the proteins of interest. The benzophenone derivative of phenylalanine p-Benzoyl-L-phenylalanine (Bpa)³, has been widely used as a photoreactive cross-linker⁴⁻⁶. This phenylalanine analog can be chemically incorporated into peptides by solid-state synthesis⁷ or genetically inserted into proteins in living cells using an orthogonal tRNA-tRNA synthetase pair which incorporates Bpa in response to a specific nonsense codon, such as the UAG amber engineered into specific sites of the target protein^{8, 9}. A Bpa-labeled protein engages in non-covalent interactions within a cell lysate or the interior of a living cell until the introduction of UV light energy into the system activates the photoreactive group, which then forms a bond to nearby interacting molecules. The resulting complexes can then be isolated and analyzed by biochemical or mass spectrometric approaches. This methodology has been used to identify active sites of enzymes¹⁰, map protein channels¹¹, study interactions in protein complexes¹², find protein interacting partners with antimicrobial peptides¹³, and examine receptor-ligand binding¹⁴⁻¹⁶.

A typical source in a UV chamber used to initiate cross-linking reactions is a 350 nm UV bulb; the recommended exposure time to activate photoreactive amino acids, such as Bpa, ranges from 30 to 120 minutes¹⁷. The exact cross-linking time varies, depending on the specific UV source, but is generally on the order of tens of minutes.^{12, 18-23}. Although in comparison to chemical crosslinking and certain other photoactive amino acid analogs Bpa is known to be relatively free from non-specific reactions, long UV chamber irradiation periods may increase the chance of side reaction and non-specific crosslinking. To interrogate transient protein interactions in living cells, it is essential to complete the cross-linking reaction on a time scale of seconds rather than minutes. One obvious means towards this end is to deliver the necessary energy on a shorter time scale. A pulsed UV laser can generate a very short burst of irradiation with extremely high peak power, much higher than would be produced if it were operated in continuous wave mode. For example, a 355 nm, 1W laser operating at 50 kHz can generate peak power in the range of 3000 W per pulse.

Unlike chamber-based irradiation where the energy is not directed onto the sample, the beam of the laser can be focused into a vessel holding the proteins of interest, ensuring the delivery of the UV energy directly to the sample.

In this report, we assessed the ability of a pulsed UV laser to stimulate the cross-linking using the model system of a Bpa-containing peptide, the tridecapeptide yeast pheromone α -factor, and bovine serum albumin (BSA). Serum albumin is a blood-borne protein which serves to bind, transport and deliver substances, including hormones and drugs, via the circulatory system²⁴. Drug-BSA interactions have been studied by variety of biochemical and biophysical methods^{25–28}. The analysis of serum albumins, including crystal structures^{29, 30}, solution structures³¹ and biochemical approaches^{32, 33} reveals the presence of seven evolutionarily conserved high-affinity binding pockets for medium and long chain fatty acids, four additional sites for short chain fatty acids, as well as numerous sites which bind drugs or other ligands. The capacity of BSA to bind hydrophobic ligands with high affinity, coupled with its well documented use in LC-MS as a model protein prompted its use as a substrate for Bpa- α -factor cross-linking.

We had previously used a biotinylated version of this α -factor synthesized with Bpa at various positions ([bioBpa] α -factor – Fig. 1) in conventional Bpa-mediated UV cross-linking to define points of contact between the pheromone and its cognate G protein-coupled receptor Ste2p^{18, 19, 34}. In addition, in the study reported herein we investigated cross-linking of BSA to the decapeptide gonadotropin releasing hormone. GnRH (also known as luteinizing hormone releasing hormone, LHRH, or follicle stimulating releasing hormone, FSHRH), is a neurohormone. It is produced in the hypothalamus and carried via the bloodstream bound to serum proteins to the pituitary gland where it binds its cognate G protein-coupled receptor. This peptide has sequence homology to α -factor and it has been demonstrated that α -factor can bind to and activate the rat GnRH receptor³⁵. The goals of our study were two-fold: (1) to determine the parameters under which UV laser energy would effectively stimulate photoactivation of [bioBpa] α -factor and GnRH cross-linking to BSA and (2) to utilize mass spectrometry to ascertain the specificity of the interaction. An outcome of this study was the determination of the site(s) of the peptide-BSA interactions.

RESULTS

Optimization of laser activated cross-linking of [bioBpa] α -factor to BSA with respect to irradiation time and energy

BSA incubated in the presence or absence of a 100-fold molar excess of [bioBpa] α -factor was irradiated for 0, 10, 30 or 60 seconds using the Explorer One XP laser at a pulse repetition frequency (PRF) of 50 kHz. The laser output at 30% and 50% of the maximum diode laser current delivering 3.9×10^{-6} J per pulse and 17.5×10^{-6} J per pulse, respectively, was assessed for the ability to promote cross-linking. Irradiated BSA samples were transferred to a membrane and probed with an IR dye conjugated to streptavidin to detect [bioBpa] α -factor cross-linked to the protein. Band density was normalized to the densest band (60s/50% laser current =100%). In the absence of laser energy, no signal was visually observed in the presence of a 100-fold molar excess of [bioBpa] α -factor [bioBpa] α -factor (Fig. 2), although the band quantitation indicated a 5% signal at the molecular weight

for the cross-linked product indicating that there was a very limited non-specific association between the peptide and BSA. Biotinylation increased as a function of time and laser output, with the highest signal intensity associated with sample irradiated for 60 s at 50% laser output. At the lowest irradiation time (10s), the band density doubled (27% to 64%) as the laser output increased from 30% to 50%. At the longest irradiation time (60s) increasing laser current from 30% to 50% resulted in a 1.4-fold increase in band density. However, as laser time and intensity increase, there was also an increase in high molecular weight forms of the BSA-[bioBpa] α -factor complex as well, suggesting an increased tendency towards BSA aggregation. An increase in low molecular weight smearing of signal also increased as a function of time and laser output, likely due to cross-linked concatamers of [bioBpa] α -factor or degradation of BSA cross-linked to [bioBpa] α -factor (Fig. 2).

Irradiating samples at 30% laser output for times as short as 1 s promoted cross-linking of the [bioBpa] α -factor to BSA, although at only 14% percent of that observed for the 30s irradiation (Fig. 3A), so cross-linking is possible on much shorter time scales, but at the expense of yield. The software indicated that the energy per pulse at a pulse repetition frequency (PRF) of 50 kHz and laser output set at 30% was 3.9×10^{-6} J/pulse. Irradiating a sample with this energy for 30 s at 50,000 pulse/sec results in the delivery of approximately 6 J. Modulating irradiation time, laser current and PRF, a constant amount of energy, 6 J in this case, can be delivered to the sample in different ways. Samples were irradiated at either 50 kHz or 200 kHz adjusting the diode current and time such that 6J of energy was delivered per sample. The intent was to determine if shorter irradiation times at higher laser fluence was more effective or less effective than longer times at reduced laser fluence. Under most conditions, it was determined that the method by which the 6J was delivered did not influence the amount of cross-linked product generated (Fig 3B) as previously shown as well in another study³⁶. There was a modest increase in cross-linking at 200 KHz PRF at the highest laser diode current (90%), but cross-linking under all other conditions was similar to that of the control. The parameter which is important is the amount of energy delivered. The 30s/30% laser output at a PRF of 50 kHz was therefore used as the standard parameters for cross-linking to maximize cross-linked product formation while minimizing the formation of intermolecular cross-linking of BSA. In comparison to the laser-induced cross-linking, no detectable cross-linking was observed using a standard UV bulb (365nm) after one minute irradiation. Increasing cross-linking time to 10 minutes was required for a barely detectable α -factor-BSA cross-linking (data not shown).

Identification of Cross-Linked Products by HPLC-MS/MS

High resolution MS measurements were carried out for the analysis of the cross-linked peptides. Bands containing BSA-[bioBpa] α -factor cross-linked material were excised from stained SDS gels and processed for HPLC-MS/MS analysis. A “high-high” MS approach with a LTQ-Orbitrap-Elite was employed to measure both the parent peptide ions and fragment ions at high mass resolution. HCD fragmentation yields higher energy MS/MS spectra and also provides the charge information of fragment ions, which makes the manual interpretation of specific MS/MS spectra much easier. Note that we are able to simultaneously monitor all digested peptides, including the majority of abundant BSA peptides that do not contain a cross-linked component. While this data is not shown, it is

essential to measure and identify all “normal” tryptic peptides to eliminate them appropriately and to observe and identify “unusual” higher charged peptides that may represent cross-linked candidates. Subsequently, cross-linked products were identified by StavroX. This software is designed for identifying cross links of peptides measured by mass spectrometry and has a Bpa setting as one default cross linker; we thus chose this software for our mass spectrometry data analysis.

Initially, BSA incubated in the presence or absence of a 100-fold molar excess of [bioBpa] α -factor under various laser conditions (irradiation times, power density) were evaluated by mass spectrometry. In order to investigate the generation of cross-linked products as a function of different laser current, four samples were examined by mass spectrometry, including controls (single BSA) and mixtures ([bioBpa] α -factor bound to BSA) under 30s/30% maximum diode current or 30s/60% maximum diode current. After cross linking, one good indicator of the presence of cross-linked peptides is high charge state ions³⁷. Under various laser energy conditions, we observed that even without Bpa, intramolecular cross-linking within BSA was present, as evidenced by the high charge state ions in single BSA samples (Figure 4(a) and 4(b)). The charge state information of all the precursor ions that was recorded in MS1 scans was retrieved by Raxport program³⁸, to explicitly illustrate the impact of different laser conditions on the formation of high charge state ions in single BSA samples. For example, precursor ions with charge state up to +4 and +5 were recorded in control BSA sample under 30s/30% conditions (Fig. 4a), while even higher charge state ions (up to +8) were recorded in the control BSA sample under 30s/60% conditions. This reveals the presence of much larger peptides (neutral molecular masses exceeding 13 KDa) in the higher irradiation samples suggesting that internal aromatic amino acids within BSA are susceptible to laser induced cross-linking and higher laser current initiated intramolecular cross-linking within BSA. This result showed also that besides the BSA aggregation observed in the gel bands (Figure 2) at increased laser output, formation of intramolecular cross-linking of BSA was also increased at higher laser output. Therefore, the protein gel data along with mass spectrometry data informed us that reduced laser output (30s/30%) would reduce the amount of undesirable inter-molecule and intra-molecule cross-linking, thus promoting only the Bpa-induced cross-linking.

Samples which have [bioBpa] α -factor bound to BSA under these two different laser outputs were also measured by mass spectrometry. Distinctly different high charge state ions were observed in [bioBpa] α -factor-BSA cross-linked samples (Fig. 4C and 4D) relative to their corresponding control BSA samples (Fig. 4A and 4B), revealing the presence of cross-linked BSA and [bioBpa] α -factor constituents. When searched with StavroX, multiple BSA peptides were found to be cross-linked to [bioBpa] α -factor, which suggests two possibilities. One is that BSA has multiple binding sites for [bioBpa] α -factor. The other possibility is that 100-fold molar excess of [bioBpa] α -factor allowed for random cross-links to residues of BSA. In order to minimize random cross-linking, the molar ratio of BSA to [bioBpa] α -factor was reduced sequentially to 1:50, 1:25, 1:12.5, 1:6.25 in order to identify the binding site with highest affinity. Protein blot results showed that signal intensity increased as a function of the amount of [bioBpa] peptide present, although there was no difference between 6.25 and 12.5-fold excess (Fig. S1). Considering that as low as 6.25-fold molar excess of [bioBpa] pheromone still yielded significant cross-linked product (Fig. S1) and it

is easier to identify the unambiguous cross-linked BSA peptides and [bioBpa] α -factor at lower molar ratio, the two lowest molar ratio samples (where BSA: [bioBpa] α -factor is 1:12.5, 1:6.25) were selected and further measured in mass spectrometry. Additionally, cross-linking with a 10-fold excess of [bioBpa] α -factor under different laser conditions was also measured. We anticipated that the BSA peptide which contains the binding site of highest binding affinity would be consistently identified in these different molar ratio samples and under several different laser conditions.

Mass spectrometry results revealed that two tryptic peptides of BSA were consistently identified as cross-linked to [bioBpa] α -factor in these samples (Table 1): Peptide 1 - HPYFYAPELLYYANKYNGVFECCQAEDKGACLLPKIETMREK and Peptide 2 - DDSPDLPKLKPDPTLCDEFKADEKKFWGK. Note that this table reveals distinct versions (i.e., different lengths and charge states) of the same basic two peptide regions. The 3D structure of BSA revealed that these two regions are spatially very close. When reducing the molar ratio of BSA to [bioBpa] α -factor to 1:6.25, Peptide 1 (HPYFYAPELLYYANKYNGVFECCQAEDKGACLLPKIETMREK) was still identified, but not Peptide 2. This suggested that the Peptide 1 binding region, previously identified as fatty acid binding site 1²⁹, may have the highest binding affinity for [bioBpa] α -factor. However, the signal decreased dramatically at this molar ratio, making the assignment of fragment ions more difficult, resulting in a somewhat lower confidence score (11) in StavroX.

A second analysis was conducted using Crossfinder to independently validate the search results from StavroX. Crossfinder identified 6 different binding regions for BSA using the same set of samples under different conditions (Table S1). The region which had the highest score overlaps the same **Peptide 1:** (RHPYFYAPELLYYANKYNGVFECCQAEDKGACLLPKIETMREKVLASSAR) that was identified by StavroX.

Cross-linking of non-Bpa containing peptides to BSA

As mentioned above, the protein gel data and the HPLC-MS/MS results suggested that extended irradiation times at high laser current promoted cross-linking within and between BSA molecules. This raised the possibility of non-Bpa mediated cross-linking; peptides which do not contain a photoactivatable group could be laser activated and cross-linked to a protein, such as BSA. To test this possibility, a biotinylated α -factor analog lacking Bpa, [bio] α -factor (Fig. 1) was incubated with BSA and irradiated at different times and laser output levels. Band density on blots was determined and normalized to the cross-linking of [bioBpa] α -factor at 30s/30% laser output (Fig. 5). Irradiation of the samples containing [bio] α -factor did yield cross-linked product as detected by immunoblot when irradiated for either 30s or 60s at 30% or 50% laser output. The amount of product generated was reduced in comparison to irradiation of [bioBpa] α -factor at the same time and laser output, suggesting that while possible to cross-link native, biotinylated peptide to BSA, the presence of Bpa in the peptide greatly enhances product yield.

Since [bio] α -factor could be cross-linked to BSA in the absence of Bpa, we assessed the ability to cross-link GnRH, a blood-borne neurohormone with sequence similarity to α -

factor, to BSA. To determine if GnRH binds to BSA in a manner similar to that of α -factor, a biotinylated GnRH analog ([bio]GnRH) was incubated with BSA and irradiated at different times and laser outputs (Fig. 6). The results indicated that [bio]GnRH could be cross-linked to BSA. Like [bio] α -factor, the amount of cross-linked product generated depended on irradiation time and laser output. Cross-linking was low in efficiency for [bio]GnRH; at the maximum laser fluence (60s at 75% laser output) the band density was only 13% of the [bioBpa] α -factor control (30s at 30% laser output).

The possibility existed that GnRH might bind to BSA non-specifically. However, if α -factor and GnRH interact with the same binding pocket(s) within BSA, then the presence of one peptide should prevent the binding of the other, depending on the concentration of each and their respective binding affinities. To test this, cross-linking (30s/30% laser output) of [bioBpa] α -factor (30 μ M) in the presence of a 10-fold molar excess of GnRH peptide or native α -factor was measured (Fig. 7A). The reciprocal experiment, cross-linking of [bio]GnRH to BSA in the presence of a 10-fold excess of native GnRH or native α -factor was also completed (Fig. 7B). In the presence of α -factor, the cross-linking of [bioBpa] α -factor decreased to 9% of the control level, suggesting that the two peptides are binding to the same site on BSA. In contrast, GnRH reduced the signal to about 55% of the control level. While this represents reduction in [bio]Bpa cross-linking, GnRH was not as effective as α -factor at preventing the cross-linking of the [bioBpa] analog. When [bio]GnRH was cross-linked in the presence or absence of competitors, α -factor again competed very effectively for cross-linking; in this case, reducing the [bio]GnRH to 8% of the control. As noted in the reciprocal experiment, native GnRH was not as effective as α -factor at preventing the cross-linking [bio]GnRH, reducing the signal to 58% of control values. It is possible that GnRH has a lower affinity for binding to BSA, thus in comparison to α -factor, it is not as effective a competitor. The results indicate that native α -factor effectively competes with both [bioBpa] α -factor and [bio]GnRH for cross-linking to BSA supporting the hypothesis that these peptides bind to the same site(s) on BSA.

Measurement of biotinylated peptide-BSA binding affinity

A steady-state binding assay using surface plasmon resonance (SPR) was completed using [bio]GnRH, [bio] α -factor and [bioBpa] α -factor to assess their binding affinities. The peptides were immobilized via their biotin tag to the sensor chip surface and the change in resonance units (RU) was measured as BSA at different concentrations (0.03 – 1 mM) was flowed over the surface. For [bio] α -factor and [bioBpa] α -factor the K_D values for binding were $222 \pm 17 \mu$ M and $673 \pm 36 \mu$ M (Fig. 2S), respectively. In contrast, there was no detectable binding of BSA to the [bio]GnRH peptide. In [bio]GnRH biotin is located on the first residue (biotinyl-(Gln¹)-GnRH; for the biotinylated α -factor analogs biotin is located on K⁷ in the middle of the peptide. Lack of measurable binding, despite the ability to cross-link GnRH to BSA is likely due to the attachment of the N-terminus to the chip surface.

MS identified that GnRH binds to BSA at the same region as [bioBpa] α -factor

To determine the site(s) of GnRH cross-linked to BSA, mass spectrometry was employed. Cross-linked results obtained via the StavroX showed that the BSA peptide “YNGVFQECCQAEDKGACLLPKIETMREKVLASSARQR” yielded a score (76) as cross

linked to the bio GnRH. This is the same region as that identified in the BSA-[bioBpa] α -factor experiment, which supports the result of the competition experiment above. The +6 charge state of the BSA/Bio-GnRH complex at $m/z = 933.629$ was selected and fragmented by HCD and the MS/MS spectrum is shown in Figure 8. Multiple b, y ions of α peptide and β peptide were found (α peptide and β peptide are the two cross linked peptides, the peptide which has more fragments found is regarded as the leading peptide, termed as α peptide). Although the existence of y35 ion indicated that either the N-terminal Y or N amino acid of the BSA can be the crosslinking site for bio-GnRH; given the low intensity of this fragment, we did not consider it as solid enough evidence to localize the specific interaction site.

Computational DOCKing analysis

Geometry optimization using DOCK6 suggested that the bound α -factor adopted an orientation similar to its biologically active geometry³⁹ when interacting with the Ste2p receptor, such that the N-terminus (Trp1) is located deep in a fold or pore of the van der Waals surface of the protein with the rest of the ligand protruding out of the fold before making a β -turn at Pro8 so that the C-terminus projects back into the fold at roughly half the depth of Trp1. Bovine serum albumin provides the peptide ligand a scaffold to effectively replicate this geometry, with the deep cleft between domains I and III of BSA serving as the analog to the β -barrel formed by the transmembrane domains of Ste2p. DOCK6 places Trp1 in close proximity to His145 (3 Å), Ser192 (3 Å), and Pro146 (3 Å) of BSA. From Trp1, α -factor extends 21 Å out of the cleft between domains I and III until making a β -turn at Pro8 near the opening of the cleft. At this point, Pro8 is nearest to Lys116 (3 Å). From here, α -factor extends 15 Å back into the cleft so that at the C-terminus (Tyr13) is located 3 Å from Thr434 of BSA and 16 Å from Trp1 of the α -factor peptide (Fig. 9). An animated version of Figure 9 is submitted as Figure S1. All of these distances are compatible with the mass spec data, although the specific residue-to-residue interactions the peptide and BSA have not been identified in the spectra. His145 is the same His residue as in Peptide 1 (HPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMREK) as identified by StavroX and Crossfinder. This both supports the α -factor binding orientation predicted by DOCK and serves as further evidence supporting Peptide 1 as a primary binding site candidate, as all three software tools employed agree that the same subsequence likely contains the cross-linked site.

DISCUSSION

Photoactivated cross-linking of amino acid residues using UV lamps has been a very useful method for investigating protein-peptide interactions. A reagent used often for this technique is p-benzoyl-L-phenylalanine (Bpa) which forms covalent cross-links with nearby amino acids when irradiated by UV light at a wavelength of ~360 nm that is optimal for Bpa activation. When coupling high resolution mass spectrometry focused on peptide identification with the substitution of Bpa into specific sites in a peptide or protein, sites of interaction can be pinpointed. Some conventional limitations of this overall methodology have been the relatively long irradiation times (30 min as the normal UV irradiation time) and the difficulty in incorporating Bpa into cellular proteins.

To address the limitation of a long irradiation time, UV lasers have been used extensively to cross-link proteins and nucleic acids^{40–42}. By this method nucleic acid bases are excited to cross-link to nearby proteins in order to freeze interactions in real time allowing a snapshot of the steps in the assembly of such complexes. In contrast, UV lasers have been used to a much lesser extent to initiate cross-links between peptides and proteins or peptides and peptides. Perhaps the first study to use femtosecond pulsed UV lasers to cross-link peptides to peptides found by mass spectroscopy that cross-linking was mediated by aromatic amino acids and that a 260 nm UV laser did not damage the peptides under the conditions used for cross-linking⁴³. UV laser irradiation was recently applied to cross-link glyceraldehyde-3-phosphate dehydrogenase both in vivo and in vitro to form stable interunit dimers³⁶. In the study reported herein, we developed a method whereby cross-linking between a peptide and a protein was rapidly and effectively facilitated using a UV-laser pulse at 355 nm. The model system used was a Bpa-containing peptide and bovine serum albumin, a well-studied protein. Mass spectrometry provided the means to determine the specificity of the cross-linking.

The tridecapeptide mating pheromone α -factor of the budding yeast *Saccharomyces cerevisiae* binds to its cognate receptor Ste2p, a model G protein-coupled receptor (GPCR). The α -factor-Ste2p system has been used often as a model for GPCR structure and function. Biotinylated, Bpa-modified α -factor was available to us from previous studies of cross-linking this analog to Ste2p¹⁸. The biotin tag on α -factor allowed us to follow cross-linking of the peptide to BSA on protein blots in order to assess the influence of time, energy, and the presence of competitors on laser cross-linking. We emphasize that irradiation of peptides or proteins can result in the formation of crosslinks involving photoactivateable sidechains in these molecules. Among these sidechains are the aromatic groups in Trp and Tyr residues and in unnatural residues such as benzoyl-phenylalanine that is widely used in crosslinking studies^{10, 43}. Often, to facilitate crosslinking analysis, tags such as biotin are included to detect incorporation caused by irradiation. The biological activity of the biotinylated Bpa- α -factor peptide is almost identical to that of the non-biotinylated native peptide¹⁸. Biotin tags have been used previously in photocrosslinking of peptides without observation of side effects⁴⁴. Given the chemical bonds in biotin we believe that it is highly unlikely that this tag results in cross-linking artefacts. Since α -factor is hydrophobic, we hypothesized that this peptide would bind to one or more of the many hydrophobic or drug binding pockets in BSA²⁹. This was confirmed by LC-MS-MS analysis that determined [bioBpa] α -factor likely inserts into a well-defined fatty acid binding pocket in BSA, recapitulating the manner in which α -factor is hypothesized to bind to a hydrophobic pocket in its cognate receptor. The generation of a cross-linked product was dependent upon the amount of energy added to the system (Fig. 2 and Fig. 3). The interaction between [bioBpa] α -factor and BSA was specific, since cross-linking was severely reduced (9% of control levels, Fig. 7A) in the presence of native α -factor, suggesting competition for a common binding site.

BSA is routinely used as a substitute for human serum albumin (HSA) in tissue culture or other applications. There is a very high degree of sequence and structural conservation across human, bovine, equine and leporine serum albumins²⁹. Sequence identity ranges from 71–76%, and 17 conserved disulfide bridges hold the 584 amino acids into a three-domain structure. Thus the information obtained from modeling of [bioBpa] α -factor cross-

linking to BSA studies can likely be generalized to cross-linking of peptides, drugs or other compounds to serum albumins, including HSA.

Benzophenones preferentially abstract hydrogen from sterically accessible C-H bonds, within the reactive distance of approximately 3 Å, resulting in recombination of the radical to form a covalent bond⁴⁵. Bpa preferentially cross-links to C-H bonds which form stabilized free radical intermediates such as those from amino acids with CH₂ groups adjacent to heteroatoms (Met, Lys, Arg) or with the tertiary C-H bond (Leu, Val)⁴⁶. Methionine is often identified as the site of cross-link formation, owing to the presence of the adjacent sulfur heteroatom which stabilizes the intermediate radical⁴⁵. A methionine “magnet effect” has been described, which suggests that the specific site of cross-linking between a Bpa-containing peptide and its binding partner can be influenced by the presence of methionine, as demonstrated by scanning methionine mutagenesis of a ligand binding pocket. The BSA peptide associated with [bioBpa]α-factor post-laser irradiation H¹⁴⁵PYFYAPPELLYYANKYNGVVFQECQAEDKKGACLLPKIETMREK¹⁸⁷ contains a single methionine residue at position 184. The computational DOCKing analysis of the interaction between [bioBpa]α-factor and BSA places the Bpa¹ residue in close proximity to H¹⁴⁵ and P¹⁴⁶ of BSA, thus it is unlikely that methionine influences the binding.

While optimizing the cross-linking conditions, we also determined that the delivery of large doses of energy to the system resulted in a high degree of intramolecular cross-linking within BSA itself, evidenced by resistance to trypsin digestion while preparing samples for MS analysis (data not shown). This observation suggested that native amino acids can form radicals upon laser irradiation of sufficient intensity resulting in the formation of stable, covalent cross-links. Laser irradiation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) both *in vivo* and *in vitro* was recently reported to form stable dimers via intersubunit cross-linking under laser conditions similar to those reported in this study³⁶. These observations prompted us to determine whether laser energy could cross-link a non-Bpa-containing peptide to a binding site within BSA, thereby mimicking receptor-ligand binding and extending the applicability of laser cross-linking to protein partners lacking photoactivatable groups. Therefore, we examined [bio]α-factor, an analog lacking Bpa, but otherwise identical to [bioBpa]α-factor and found that this peptide could be cross-linked to BSA, although with much lower efficiency than its Bpa-containing counterpart (Fig 5).

SPR measurements to assess the binding of [bio]α-factor to BSA compared to that of [bioBPA]α-factor indicated that the affinity of biotinylated peptide was 3-fold higher ($222 \pm 17 \mu\text{M}$) than that of [bioBpa] α-factor ($673 \pm 36 \mu\text{M}$). This level of binding is reasonable for interaction of a small, hydrophobic peptide to a hydrophobic pocket in a protein. Despite its reduced binding affinity, when laser cross-linking of [bioBPA]α-factor was undertaken, the presence of Bpa enhanced the UV-mediated association in the peptide with BSA over that observed for [bio]α-factor. During the course of the cross-linking experiments, the amount of peptide used ranged from 6- to 100-fold molar excess ($= 0.25 \mu\text{M} - 150 \mu\text{M}$) relative to BSA, concentrations which are well below the apparent K_D value of $673 \mu\text{M}$ for [bioBpa]α-factor. Given the fact that the binding site is not saturated it is likely that the ligand is binding to a discrete site on BSA.

We extended the utility of this approach to the vertebrate hormone GnRH. There are multiple distinct isoforms of GnRH, but all are decapeptides. GnRH1, used in this study, plays a key role in regulation of reproductive processes in vertebrates^{47, 48}. The peptide sequence (PyrHWSYGLRPG) is invariably conserved across a mammalian species and very highly conserved across vertebrates^{49–51}. In mammals, GnRH is secreted from the hypothalamus into the pituitary portal system where it binds G protein-coupled receptors (GPCR) in the adenohypophysis, resulting in the secretion of the gonadotrophic hormones LH (luteinizing hormone) and FSH (follicle stimulating hormone). Remarkably, this vertebrate reproductive hormone shares 80% sequence homology with the N-terminal nine residues of α -factor, the pheromone ligand of the yeast mating GPCR (Fig. 1)⁵². The homology between these two eukaryotic reproductive hormones is sufficient to allow the yeast peptide to activate the rat GnRH receptor³⁵. As we observed for bio α -factor, a biotinylated variant of GnRH also cross-linked to BSA at higher laser output (Fig. 6) and cross-linking was reduced in the presence of α -factor or native GnRH (Fig. 7B), suggesting competition for a common binding site. Interestingly, GnRH was not as effective as blocking the cross-linking of [bioBpa] α -factor to BSA as α -factor was at blocking [bio]GnRH cross-linking. This suggests that α -factor has a higher affinity for BSA than does GnRH.

Despite the fact that [bio]GnRH could be cross-linked to BSA in a manner that suggests it shares a common binding site with α -factor, the binding affinity of [bio]BSA could not be determined by SPR under the conditions used. The absence of binding is likely related to the location of the biotin tag on the N-terminal glutamine residue of the peptide. During the SPR measurements, the peptide is immobilized to the surface of the chip via the biotin tag. For the α -factor analogs, biotin is attached to the side chain of position 7, which is at the apex of the hairpin turn in the molecule. This allows free access for both the N- and C- termini of α -factor to interact with BSA, as they do when binding to their cognate receptor³⁹. For the [bio]GnRH analog, biotin capture to the chip resulted in fixation of the N-terminus to the chip, blocking any potential interaction of the N-terminus. Comparison of the sequences show that both GnRH and α -factor have significant hydrophobicity, HisTrpSerTyr and HisTrpLeu, respectively, at the N-terminus which is likely necessary for binding to BSA. This observation lends further support to the hypothesis that binding of GnRH and the α -factor analogs is a specific interaction within a binding pocket in BSA and reflects the normal binding of α -factor to its receptor.

Mass spectrometry has become one of the primary methods to characterize molecular interactions, and even molecule structure and function. In recent years, integrations of crosslinking experiment and mass spectrometry measurement has become an important technique to identify protein interactions *in vivo* or *in vitro*⁵³. In this study, we adopted a “high-high” MS approach with an LTQ-Orbitrap-Elite to measure both the parent peptide ions and fragment ions at high mass resolution. Fragmentation was accomplished with an HCD (Higher Energy Collision Dissociation) approach. This HCD fragmentation is superior to CID fragmentation, especially for large cross-linked peptides in providing more efficient fragmentation and more distinct MS/MS spectra. By examining a range of possible high-charged peptides, we found multiple lines of evidences pointing to the same binding region of BSA to both peptides.

In this study we have demonstrated the utility of laser-activated cross-linking of Bpa-containing peptides and, to a lesser extent, the ability of laser to cross-link native amino acids. We believe the methodology can be extended to capture protein interactions in a live cell using unnatural amino acid incorporation of Bpa into proteins in the native state^{8, 9, 54}. The ability to capture such interactions in the living cell in a very short time using a laser should allow “real-time” analysis of protein interactions of intracellular pathways and processes.

EXPERIMENTAL PROCEDURES

Reagents

Nle¹²α-factor (α-factor, Fig. 1), Bpa¹K⁷(bio ACA)Nle¹²α-factor ([bioBpa]α-factor, Fig. 1) and K⁷ (bio ACA)Nle¹²α-factor ([bio]α-factor) were prepared as previously described¹⁸. L-norleucine (Nle), which is isosteric with L-methionine, was incorporated at position 12 in all synthetic α-factors to replace the naturally occurring L-methionine. This prevents oxidation of the sulfur atom of methionine during peptide synthesis and purification. Replacement of Met with Nle was shown previously to result in an analogue with activity and receptor affinity equal to those of the native pheromone⁵⁵. BSA (Fraction V) was obtained from Research Products International (Mt. Prospect, IL). GnRH (also known as LHRH, Fig. 1) peptides (LHRH Acetate Salt and Biotinyl-(Gln¹)-LHRH trifluoroacetate salt) were purchased from Bachem Americas Inc. (Torrance, CA). Peptides were prepared as aqueous stocks and stored frozen in small volumes to minimize repeated freeze-thaw events. Phosphate buffered saline (PBS: 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 3 mM KCl, 150 mM NaCl, pH 7.4) was prepared from analytical grade reagents.

Laser Cross-Linking

One hundred microliters of BSA (0.1 μg/μl in PBS) was combined with the various peptides in the molar ratios specified for each experiment and dispensed into a 2.0 ml Eppendorf tube kept chilled on ice. The Explore One XP 355-1 UV laser (Spectra-Physics, Santa Clara CA) controlled by L-Win, a LabView-based graphical user interface, was used to irradiate samples. Unless otherwise specified, pulse repetition frequency was 50 kHz, the diode current was set to 30% of the maximum output (5.49 A), and the irradiation time was 30s. The beam was directed through a plano-concave lens (f 75.0 mm, Ø1” UV Fused Silica, Thorlabs, Newton, NJ) to expand the diameter to approximate that of the diameter of the sample tube (10 mm), and reflected off a fused silica UV-laser mirror (Thorlabs, Newton, NJ) down into the tube. To begin the irradiation, the tube containing the sample was placed into a benchtop freezer block, chilled to -20C, the block positioned such that the aperture of the tube was in alignment with the beam, and the samples were irradiated. Upon completion of the irradiation interval, samples were mixed with 50 μl 3X SDS sample buffer (30% glycerol, 15% β-mercaptoethanol, 3% sodium dodecyl sulfate or SDS, 0.01% bromophenol blue, 0.1875 M Tris, pH 6.8) and stored at -20C until used. Rapid laser-induced labeling under laser irradiation conditions does not alter the native protein structure or interactions, as has been shown for numerous laser-induced covalent labeling applications⁵⁶.

SDS PAGE and Protein Blotting

Samples (1.0 µg BSA per lane) were fractionated by discontinuous SDS PAGE (5% stacking gel, 10% separating gel). For MS analysis, gels were stained (50% methanol, 10% acetic acid, 0.2% Coomassie Brilliant Blue R-250) for at least one hour and destained (10% acetic acid, 10% methanol, 2% glycerol) overnight. The bands were excised and placed into Eppendorf tubes, washed 3 times with distilled water and stored without liquid at -20°C until used for MS analysis. Multiple lanes were run in parallel and bands were pooled to provide sufficient material for MS analysis.

For detection of BSA cross-linked to a biotinylated peptide the laser cross-linked BSA samples were transferred to Immobilon-FL PVDF membrane (Millipore, Billerica, MA). The membranes were allowed to air dry at room temperature, re-wetted in Odyssey Blocking Buffer and processed for detection (IRDye 800CW Streptavidin conjugate) using the Odyssey CLx Imaging System (Buffer, Dye conjugate and Imaging System, LI-COR Biosciences, Lincoln, NE) as specified by the manufacturer. Band intensity was measured using the Image Studio 4.0 software (LI-COR Biosciences).

Protein extraction and proteolytic digestion

Protein extraction from the gel bands and subsequent digestion was performed by following standard in-gel digestion protocol for mass spectrometric characterization of proteins⁵⁷. Briefly, the bands were rinsed with two successive additions of 1 mL water before being crushed with a pestle in 100 µL of 50 mM acetonitrile/ 50 mM ammonium bicarbonate (50:50, v/v). An additional 500 µL of 100% acetonitrile was added to dehydrate and shrink the pulverized gel fragments, then as much liquid as possible was removed without disturbing any of the gel fragments. Twenty µg of porcine trypsin (Sigma Aldrich, St. Louis, MO) was dissolved in 1.5 mL of a buffer solution consisting of 10 mM ammonium bicarbonate (pH = 7.5) and 10% acetonitrile (50:50, v/v). This solution was added to the crushed, dehydrated gel pieces until the fragments were entirely submerged (~50 µL). The gel fragments were rehydrated at 4°C for 2 hours before being placed in a 37°C water bath to digest overnight. The following morning, an extraction buffer containing 100% acetonitrile + 5% (vol/vol) formic acid was prepared, and was added to the digested peptide samples in a ratio of 1:2 sample volume: extraction buffer. After a 15 min incubation period at 37°C, the solution was filtered through a 10 kDa molecular weight cutoff spin column filter (Vivaspin 2, GE Healthcare Life Sciences, Pittsburgh, PA) to recover the peptides for final clean up.

Surface Plasmon Resonance determination of binding of peptides to BSA

Steady-state affinity analysis of BSA binding to the biotinylated peptides was completed using the Biacore 3000 system. The Biotin CAPture Kit (GE Healthcare, Piscataway NJ) was used according to the manufacturer's instructions to immobilize biotinylated peptides (100–200 RU) to the sensor chip surface and to regenerate the chip upon completion of the assay. All assays were performed using HBS-P buffer (GE Healthcare, Piscataway NJ) as both the running buffer and diluent for the BSA analyte and biotinylated peptides. BSA analyte at different concentrations (0.03 mM – 1 mM) was injected over the immobilized peptide at a rate of 5 µl/min for 10 minutes, followed by a wash phase (5 µl/min HBS-P

buffer for 5 min) and by a dissociation phase (30 μ l/min HBS-P buffer for 10 min). Each measurement was made in duplicate and upon completion the chip was regenerated as specified by the manufacturer. Each experiment was repeated a minimum of two times. K_D values were calculated by non-linear curve fitting using GraphPad Prism 7.00 (GraphPad Software, La Jolla, CA) and are reported as mean $\pm$ sd averaged from all determinations.

Nano-LC-MS/MS Analyses

After trypsin digestion, the peptide fragments were separated by HPLC. Samples were pressure-bomb loaded onto a trap column (150 μ m i.d.), consisting of 2cm reversed phase (C18) resin (Aqua, Phenomenex) and washed offline with 100% solvent A for 20 minutes. The separation of peptides was later carried out on an in-house pulled analytical column (100 μ m i.d.), packed with 12cm reversed phase (C18) resin (Aqua, Phenomenex) with gradients from 0 to 50% B (45 min); 50% to 100% B (5min); 100% B (7min). LC solvents consisted of A: 95% H₂O, 5% ACN, 0.1% FA, and B: 30% H₂O, 70% ACN, 0.1% FA.

The analytical column was interfaced to a hybrid LTQ Orbitrap Elite mass spectrometer (ThermoScientific, Waltham, MA) operating in a data-dependent mode. Each full scan acquired in the Orbitrap over the mass range of m/z 400–1700 at $R=30,000$ ($m/z=400$), was followed by HCD (Higher-energy collisional dissociation) fragmentation of the 10 most abundant ions at normalized collision energy of 30. HCD was used to achieve higher energy dissociation for the higher charge state ions. Fragment ions were detected in the Orbitrap at a resolution of 15,000 (at m/z 400), which was essential to identify the higher charge state products. Precursor ions with unassigned charge or charge state 1 were rejected for further fragmentation. Dynamic exclusion was enabled to deepen the measurement, with the following settings: repeat count was 1, the repeat duration was 30 s, and the exclusion duration was 60s.

Identification of Cross-Linked Products

MS/MS spectra were searched against the database which contains the amino acid sequence of BSA and [bioBpa] α -factor peptide or biotinylated GnRH peptide. StavroX (version 3.6.0) was used for an automated comparison of MS and MS/MS data and identification of cross-linked products⁵⁸. Precursor ions were compared with theoretical masses of cross-linked products with mass accuracy precision set at 10 ppm and signal/noise ratio at 2:1. Fragment ions were identified with precision at 0.1 Da. Bpa was set as the cross linker with no mass loss and no preference of cross linking to any amino acid. For GnRH cross linking experiments, biotin was set as the non-specific cross linker. Oxidation of methionine was set as a variable modification. Up to six missed cleavages were allowed for Arg and Lys.

Crossfinder (Version 1.0, OMICtools, Rouen, France) was also utilized for searching the cross-linked products, to increase the confidence of the results⁵⁹. Precursor ions were compared to theoretical cross-linked product masses with precision set to 10 ppm. Fragment ions were identified and scored with precision at 50 ppm. Bpa was chosen as the non-specific cross linker with no mass loss. Oxidation of methionine was allowed as a variable modification. Up to 6 missed cleavages were allowed for arginine and lysine. The top 30 ions in each MS2 were considered for scoring, only spectra in which 3 residues or more

could be identified were considered for scoring, and only MS2 spectra whose total ion chromatogram constituted at least 5% of the total TIC of their associated MS1 were considered for scoring. Only candidates with scores greater than 100 were retained and presented in the final summary.

Computational prediction of BSA and alpha-factor binding

UCSF DOCK (version 6.7) and UCSF Chimera (version 1.11.2) were employed to both provide a visual model of alpha-factor bound to BSA and to aid in predicting the most likely binding site of the ligand^{60, 61}. The docking procedure followed previously reported protocols and parameters⁶². In brief, an x-ray crystallographic model of dimeric BSA (PDB accession number: 4F5S) with bound triethylene glycol was downloaded using Chimera's built-in structure retrieval tool²⁹. The preexisting ligand and all hydrogens were removed, and the model was made monomeric by removing one of the pair of BSA molecules. A model of α -factor was separately created with Chimera's peptide builder tool. Due to software constraints regarding unnatural amino acids, Trp was substituted for Bpa in the virtual peptide used for binding-site prediction. The van der Waal's surface of the protein was generated by Chimera and used by DOCK to determine potential binding sites. The α -factor peptide was designated as a flexible ligand, allowing DOCK to alter bond angles to find the lowest-energy orientation for binding. A scoring grid was calculated for the largest candidate site before being passed to the dock6 program for final energy minimization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Bpa	p-benzoyl-L-Phenylalanine
MS	mass spectrometry
GnRH	gonadotropin-releasing hormone
BSA	bovine serum albumin
UV	ultraviolet
Bio	Biotin
kHZ	kilohertz
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

HPLC high-performance liquid chromatography

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

Table 1

Peptides of BSA that are cross linked to α -factor as identified by StavroX.

Molar Ratio (BSA: α -factor)	Laser Power	Possible cross-linked peptide of BSA	m/z	charge	StavroX score
1:6.25	30%/30s	HPYFYAPELLYYANKYNGVFQECQAEDKGACLLPKIETMREKVLASSARQRLRCASIQK	901.958	10	11
	30%/10s	DDSPDLPKLKPDPNTLCDEFKADEKKFWGK	1109.966	5	56
1:10	50%/7s	DDSPDLPKLKPDPNTLCDEFKADEKKFWGK	1109.966	5	61
	90%/5s	FWGKYLYEIARRHPYFYAPELLYYANKYNGVFQECQAEDKGACLLPKIETMREK	1249.63	7	59
1:12.5	30%/30s	HPYFYAPELLYYANKYNGVFQECQAEDKGACLLPKIETMR	1381.074	5	60
		VASLRETYGDMADCCKEQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEK	1198.715	7	54