Development of an enrichment method for azide-containing peptides to study proteome dynamics by mass spectrometry

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Chapter 2

Development of a covalent solid phase enrichment method to isolate and study rare peptides by mass spectrometry

Abstract

To study proteome dynamics by mass spectrometry isolation of peptides of interest is necessary. In this chapter the development of a covalent solid phase enrichment method to selectively isolate azide-containing peptides is described. Different solid supports, cleavable linkers and reactive groups have been used to synthesize different types of resin. Peptides containing the unnatural azide-containing amino acid azidohomoalanine (azhal) derived from different origin were used to test the compatibility and applicability of the methods. The method should selectively enrich the peptides of interest, yielding a single product which can be analyzed by mass spectrometry, which is illustrated by the different methods developed in this study. The resin that was found to selectively enrich azide-containing peptides and allowed identification of the enriched peptides consists of poly-dimethylacrylamide as solid support, a disulphide linker that can be cleaved upon reduction and a cyclooctyne as azide-reactive group.
Introduction

To study proteome dynamics by mass spectrometry isolation of peptides of interest is necessary, as has been discussed in Chapter 1. In short; after obtaining a protein mixture or protein (complex), proteolytic treatment will deliver a complex peptide mixture that is analyzed by mass spectrometry. As only part of the peptide mixture contains the relevant information, a method has to be developed to enrich the peptides of interest. In Chapter 1 it has been described how distinction can be made between different peptides, e.g. by introduction of an amino acid containing a bio-orthogonal chemical group such as an azide-moiety. In the following two chapters (Chapter 2 and 3) different methods for the isolation of labeled peptides have been developed and will be discussed. In this chapter the development of a covalent solid phase enrichment method is described.

For the enrichment of specific peptides from a complex peptide mixture several requirements have to be met of which the most important are that the method should selectively enrich the peptides of interest yielding a single product which can be analyzed by mass spectrometry. Methods that described enrichment of peptides by covalent capturing on a solid phase are limited in number. One example is the solid-phase isotope tagging method described by Zhou et al., directed toward cysteinyl peptides. A thiol reactive group is attached via a photocleavable linker to glass beads and an ICAT-based isotope tag has been included for quantification. Galactose-induced changes in protein abundance in yeast were studied, and with this enrichment method almost exclusively cysteinyl peptides were recovered. A second example is the recently described method by Foettinger et al., in which tryptophan containing peptides are captured on hydrazine beads.

In this project, peptides containing an azide-group have to be enriched. This requires a reactive group that selectively reacts with the azide, capturing the peptides of interest on a solid phase. Furthermore, a cleavable linker is needed allowing liberation of the peptides from the solid phase. For enrichment by covalent capturing on solid phase an appropriate resin has to be selected. In this study, different types of solid supports, cleavable linkers and reactive groups have been explored in order to develop a method to enrich azide-containing peptides.

As a solid support many different types of resin are described and (commercially) available. To isolate peptides, the solid support should be
Covalent solid phase enrichment method

compatible with aqueous conditions and not release molecules interfering with mass spectrometric analysis. It should furthermore be stable under the reaction conditions and should have as little nonspecific interaction with peptides as possible. The most common solid phase for peptide synthesis and isolation of peptides is the polystyrene-poly ethylene glycol graft copolymer (PS-PEG) resin. Consisting of a polystyrene core, this resin is very stable and has good swelling properties allowing facile entrance of reagents and solvents. The poly ethylene glycol-matrix makes the resin more hydrophilic, therefore suitable to use in presence of water and peptides. A second suitable solid support may be the controlled pore glass (CPG) beads. Consisting of glass, silicium oxide, this solid phase is rigid and inert, stable under many different conditions, and compatible with water and peptides. The last solid support tested in this study is the poly-dimethylamide (PL-DMA) resin, first described in 1975 as an alternative for styrene-based resins in peptide synthesis. The resin is very hydrophilic, thus compatible with polar solvents such as water, and the polyamide backbone has similar solvation properties as peptides, therefore expected to have very little nonspecific binding of peptides.

In our approach we want to selectively isolate azide-containing peptides. The azide moiety does not react within the cell or under physiological conditions with other molecules present in the cell, but can be selectively modified by different chemical reactions, such as the (traceless) Staudinger ligation, Cu(I)-catalyzed (3+2)-cycloaddition with terminal alkynes and the strain-promoted (3+2) cycloaddition with cyclooctynes. In this study the last two types of reactions have been explored. After covalent binding of the peptides of interest to the solid phase, they have to be released again to be analyzed by mass spectrometry. In solid phase peptide synthesis different types of cleavable linkers are used, of which acid labile linkers are most commonly. The use of volatile acids such as trifluoric acid (TFA) allows easy removal of excess of reagents by evaporation. In this study, two different types of acid labile linkers were tested, the Rink amide (RAM) linker, both cleavable with 95% aqueous TFA. As an alternative for these harsh cleavage conditions, a disulphide linker cleavable under reducing conditions, has been tested.

To test the compatibility and applicability of the different methods described above for enrichment of azide-containing peptides, they have been applied to i) the azide-containing amino acid azidohomoalanine (azhal), containing a Fmoc-group, ii) model peptides, containing a single azhal residue, iii) a protein digest.
of Photoactive Yellow Protein (PYP), in which the methionine residues have been replaced by azidohomoalanine (azhal), and iv) an azhal-labeled \textit{Escherichia coli} proteome.

**Results and discussion**

**Synthesis of a solid support for covalent capturing of azide-containing peptides**

**Synthesis of PS-PEG resin**

The synthesis of PS-PEG based resin started with PS-PEG resin Hypogel RAM 200, a PS-PEG resin derivatized with an Fmoc-protected acid cleavable RAM-linker. First, deprotection of the amine was carried out by incubation of the resin with 20\% piperidine in DMF. To minimize the use of the scarce cyclooctyne moieties, coupling of a benzoic type acid to the amine of the linker was first optimized using Fmoc-(4-aminomethyl)-benzoic acid (Fmoc-4-Amb-OH) as a model compound. The carboxylic acid was coupled to the resin-bound amine by activation with a solution of HATU (O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate)\textsuperscript{26} and diisopropyl-ethyl-amine (DIPEA). This yielded the desired resin 1 in good yields (see Scheme 1).

The amount of reactive groups on the resin was determined by base induced cleavage of the Fmoc-group to liberate the fluorenyl group which strongly absorbs at 300 nm. Under standard reaction conditions an excess of acid and HATU coupling reagents is used compared to the resin, to assure complete

![Scheme 1](image-url)
coupling. However, as we want to limit the loss of the precious cyclooctyne during coupling, a slight excess of resin compared to the acid was used as well to compare yields (Scheme 1 and Material and Methods for details). After washing and drying of the beads, the loading was determined to be 0.46 mmol/g (92%) for coupling the reactive group under standard conditions, and 0.22 mmol/g (50%) for the alternative coupling conditions. For our purpose a loading of 0.22 mmol/g is sufficient: the amount of azide-containing peptides in a proteome is expected to be at least two orders of magnitude less.

**Synthesis of cyclooctyne derivatized CPG beads**

Aminopropyl CPG beads were derivatized with the acid cleavable PAL linker, after which the fluorinated cyclooctyne moiety was coupled as described for the PS-PEG resin and resin 2 was obtained with a loading of 0.17 mmol/g (Scheme 2).

**Synthesis of the cyclooctyne derivatized PL-DMA resin**

Synthesis of the cyclooctyne derivitized resins started from the commercially available poly-dimethylacrylamide (PL-DMA) resin. A cleavable disulphide bond linker was introduced by reaction of cystamine with the methyl ester on the PL-DMA resin, yielding an amide bond. Coupling of the cyclooctyne to the amine of the linker was accomplished by activation of the acid moiety using the uronium/guanidinium type peptide coupling reagent HATU, as described for the PS-PEG and CPG resin, obtaining the cyclooctyne derivatized resins: azide-reactive cyclooctyne (ARCO) resin (3a), F-ARCO resin (3b) and DIMAC-resin (3c) (Scheme 3).

The amount of reactive groups on the resin was determined after reaction of the beads with Fmoc-azidohomoalanine followed by an Fmoc assay as described.
for the PS-PEG resin. After washing and drying of the beads, similar loadings for the three types of resin were achieved, ranging from 0.15 to 0.25 mmol/g. The efficiency of the cleavage of the disulphide linker by reduction was determined using beads which were reacted with Fmoc-azhal. Incubation with different reducing agents at different concentrations and incubation times showed the reduction to be most effective using 5 mM TCEP for 1 hour at room temperature at pH 7.5, by which >90% of all Fmoc-azhal groups were cleaved from the resin.

**Cu(I)-catalyzed (3+2)-cycloaddition induces oxidation of peptides**

Before coupling the reactive groups to the solid support, their reactivity towards azide-containing peptides was investigated. For our application, it is most important that the reaction of the reactive groups with the azide-containing peptides give a clean and selective reaction. The speed by which the reaction takes place is of minor importance.

The click-reaction using terminal alkynes and Cu(I) was found to cause Cu(I)-induced oxidation of peptides, which might hamper identification. Cu(I)-catalyzed cycloaddition of 2-ethynyl-pyridine to a model peptide resulted in the observation of various oxidation products, as depicted in Figure 1. To prevent oxidation of the peptide the ligand tris-(benzyltriazolylmethyl)amine (TBTA) coordinating the free Cu(I) could be added to the reaction mixture. However, this would require removal of excess of this regent and remaining compound might interfere with mass spectrometric analysis.

As an alternative to the Cu(I) catalyzed cycloaddition, the strain-promoted
cycloaddition of cyclooctynes to peptides described by the group of Bertozzi was investigated\textsuperscript{29-32}. Reaction of a model peptide with the three cyclooctyne moieties (a-c in Scheme 3) showed for all compounds a clean and selective reaction (results not shown). For the fluorinated-cyclooctyne it was observed that the fluorine was exchanged for a hydroxyl group after formation of the triazole\textsuperscript{14}, thus yielding two products. Reaction of the model peptide with the recently described – water soluble – aza-cyclooctyne DIMAC\textsuperscript{22} showed besides the expected product an unidentified side-product. This might be caused by impurities left in the preparation, as the free acid (the last intermediate before the final step in the synthesis) is difficult to purify and synthesis was continued with the product as it is. After coupling of the aza-cyclooctyne to the resin, however, any possible contamination will be lost and no side-products are expected.

**Enrichment by PS-PEG resin and CPG beads is not compatible with reaction conditions**

As described in the introduction, an enrichment method for peptides to study proteome dynamics should yield a single product which can be analyzed by mass spectrometry. Unfortunately, for the PS-PEG solid support and the CPG beads, the reaction conditions for capturing and release of azide-containing peptides were not to be compatible with mass spectrometric analysis. Treatment of the PS-PEG resin by 95% aqueous TFA showed release of PEG’s as depicted in the MALDI-TOF spectrum in Figure 2a. It was therefore chosen not to continue with this resin, as the leached PEGs interfere with the mass spectrometric analysis and no results could be obtained.

![Figure 1](image)

**Figure 1**, Cu(I) catalyzed 1,3-dipolar cycloaddition of 2-ethynyl-pyridine to a model peptide. In time, an increase in oxidation products can be observed. a, t = 0 min. b, t = 10 min. c, t = 30 min. d, t = 60 min. e, sequence of the model peptide. X: azhal. ♦: loss of N\textsubscript{2} and uptake of 2 H. *: oxidation products (+16 Da).
Combining the fluorous cyclooctyne with the CPG beads was found to be unsuccessful due to i) unwanted side reactions after triazole formation of the azide-containing peptides and the cyclooctyne and ii) nonspecific binding of peptides to the beads. After incubation of the CPG beads with model peptides containing a single azidohomoalanine (azhal) residue (Figure 2e), the flow through was removed and the beads were thoroughly washed to remove any unbound peptides. Incubation of the beads by 95% aqueous TFA should cleave the PAL linker and release covalent captured peptides.

However, the cleavage product showed no peptides in de MALDI-spectra. On the other hand, analysis of the flow through revealed the presence of: i) unmodified peptide, ii) peptides modified by the linker, to which the peptide is bound via a triazole, attached to sillicium oxide and iii) peptides modified by both the linker and one unreacted linker, attached to the first via two sillicium oxide moieties (see Figure 2b-e). Apparently, cleavage from the beads had occurred under the experimental conditions. In addition, the flow through contained model peptide that was not modified, presumably resulting from nonspecific binding to the beads. Treatment of the flow through with 95% aqueous TFA showed two products in the spectrum: i) unmodified model peptide and ii) the expected product after enrichment, with addition in mass of 259.1 Da (Figure 2d).

Figure 2. Reagents for enrichment have to be compatible with each other and mass spectrometric analysis. For the PS-PEG resin, after cleavage of the linker with 95% TFA, leakage of PEGs was observed. For the CPG beads, the fluorine of the reactive group was released after formation of the triazole, attacking the silicium oxide of the glass beads, releasing nonspecifically the linker with attached peptide. In this figure the MALDI-TOF spectra can be found of: a, PS-PEG resin 1 treated with 95% TFA for 2 minutes at room temperature. b, model peptide, c, fluorine released peptide from CPG beads 2. An addition in mass of 660.3 Da and 1302.6 Da can be observed. d, fluorine released peptide treated with 95% TFA shows an addition in mass of 259.1 Da. e, structure of released peptide from CPG beads 2 and reaction product after treatment with 95% TFA. X: azhal. ♦: loss of N₂ and uptake of 2 H. *: salt adducts.
An explanation of this could be that after formation of the triazole between the azide-containing peptides and the cyclooctyne, one of the isomers exchanges the fluorine for a hydroxyl group, as mentioned earlier. The released fluoride now attacks the silicium oxide, enabling release of the complete linker from the beads. This reaction was confirmed by incubation of the beads with low concentrations of sodium fluoride (NaF, results not shown). In addition to the fluoride-induced release of the linker from the beads, the CPG beads derivatized with the PAL linker appeared to be very hydrophobic, showing nonspecific binding of peptides from a cytochrome c digest. The controlled pored glass beads were therefore left and a more hydrophilic solid support was chosen to continue with.

**Cyclooctyne derivatized PL-DMA resins successfully enrich azide-containing model peptides**

To test the capture and release of azide-containing peptides on the three cyclooctyne derivatized PL-DMA resins 3a-c, a model octadeca peptide (Pan016) containing one azhal residue was captured and released as described in Material and methods. Upon cycloaddition, a triazole is formed and peptides are captured on the resin via their azide moiety. Reductive cleavage of the disulfide bond liberates a thiol which is alkylated by iodoacetamide. The mass of the released peptide obtained will increase with the mass of the cyclooctyne, linker and alkylating reagent used, resulting in an addition of either i) 358.2 Da, ii) 376.2 Da or iii) 385.2 Da, depending on the cyclooctyne resin used (see Scheme 3 and Figure 3).

Pan016 was captured from a solution of 50% acetonitrile and 50% 50 mM potassium phosphate buffer pH 7.5. After incubation of 24h (or 72h) at 40 °C, release of the model peptide from all three cyclooctyne resins yielded the products with expected modification after cleavage, as is shown in Figure 3. A shift in mass can be observed after capture and release of the peptide, corresponding to the mass of the linker. In addition, the typical N₂-loss and subsequent 1 or 2 proton uptake observed for azide-containing peptides, is not observed anymore after modification (see Figure 3). For all the three types of resin good quality spectra were obtained, showing – at first sight – single products. However, a closer look shows that the fluorine-cyclooctyne resin gives rise to two products. After formation of the triazole, in presence of water one of the regio-isomers replaces fluorine with a hydroxyl group, which gives a mass difference of – 2Da. This might become a problem when studying more complex samples, as the signal gets diluted to two different products.
Modification by cyclooctyne moiety makes enriched peptides more hydrophobic

After the quality of the methods was investigated with a model peptide, more complex samples were subjected to the enrichment. A tryptic digest of the Photoactive Yellow Protein (PYP), containing six methionine residues that have been replaced by azhal, was incubated with the F-ARCO- and ARCO-resin. Similar results were obtained for both resins: after enrichment only a single unmodified peptide was observed. Furthermore, all six azhal residues were found back in the list of identified peptides.

For the fluorinated cyclooctyne modified peptides both the fluorine and hydroxyl peptides were selected for fragmentation and assigned correctly by Mascot. Furthermore, the loss of either HF or H₂O is observed, which, however, does not interfere with the identification. For the plain cyclooctyne modified peptides an ion is observed in the fragmentation spectra, caused by the loss of the linker with the cyclooctyne. This ion can be used as a reporter ion and aid in data analysis as is discussed in more detail in Chapter 4 and 6.

For the reversed phase LC experiments it was observed that the peptide mixtures enriched by the ARCO- and F-ARCO-resin show a delay in elution and elute within a relatively short time using standard gradients. The modification of the peptides by the cyclooctyne and the linker makes the peptides more hydrophobic and chromatographic behavior is dominated by the cyclooctyne

![Figure 3](image-url)

**Figure 3**, Enrichment of a single model peptide shows the selective reaction and release of the peptide with the PL-DMA resins ARCO (3a), F-ARCO (3b) and DIMAC (3c) resin. A model peptide containing a single azhal residue was incubated for 24 hours at 40 °C with the resin. MALDI-TOF spectra of: a, Peptide before addition to the resin. b, Enriched peptide by the ARCO-resin c, Enriched peptide by the F-ARCO-resin. d, Enriched peptide by the DIMAC-resin. e, Structure and sequence of model peptide enriched by the F-ARCO-resin. X: azhal. ♦: loss of N₂ and uptake of 2 H. *: salt adducts. ▲: HO-cyclooctyne modified peptide. ▼: F-cyclooctyne modified peptide.
group. Changing the gradient from linear to a combination of a linear and a step-gradient improved the mass spectrometric analysis enormously; many more ions were selected for fragmentation and the number of peptides identified by Mascot is increased as well. However, the hydrophobic modification might induce loss of material during the procedure and care should be taken to keep the enriched material in solution (see Chapter 4 and 5). The use of the more hydrophilic azacyclooctyne DIMAC as reactive group improved the elution of the peptides enormously, as was observed for enriched peptides derived from an *E. coli* proteome (see next section).

**Identification of F-ARCO and DIMAC enriched peptides from an *Escherichia coli* proteome is hampered by fragmentation side reactions**

We next demonstrate selective sequestration from complex peptide mixtures of azhal-containing peptides obtained after *in vivo* labeling of proteins synthesized by *E. coli*. For these experiments, *E. coli* was grown with azhal for two hours as described in *Material and methods*. A tryptic digest of the *E. coli* proteome was then incubated with the three cyclooctyne derivatized resins. The obtained enriched peptide mixture was subsequently analyzed by LC-MS/MS.

As discussed in previous sections, enrichment with the F-ARCO resin yielded two different types of modified peptides. And although it did not hamper peptide identification for a protein digest, data analysis with Mascot of an enriched labeled proteome yielded no peptide (and protein) identifications. The exchange of the fluorine for a hydroxyl group was however not problematic, but an (unexpected) fragmentation process hampered the analysis. In the mass spectrometer – upon fragmentation – the loss of either HF or H$_2$O, depending on the nature of the starting product, was observed (Figure 4). This is a dominant process, yielding a stable conjugated system. As peptide backbone fragmentation mainly takes place after this loss, data analysis by the data analysis program Mascot fails to match the combination of the mother-ion and the daughter-ion series to the database and only a few peptides could be identified.

Solution of this problem could be the forced loss of HF or H$_2$O by increasing the cone voltage, which would potentially yield single products. However, increasing the cone voltage might also induce peptide fragmentation, yielding a mixture of peptide products, hampering identification. A second solution would be the design of a different data analysis program, by which a difference in mass between the mother- and daughter-ions can be set. This would allow correct assignment of the daughter-ion series in combination with its mother-
ion. However, no expertise on this subject was present and the availability of alternative cyclooctynes allowed the choice for a different reactive group.

As an alternative to the more hydrophobic cyclooctynes, the recently described azacyclooctyne DIMAC was used as a reactive group\textsuperscript{22}. Comparison of the chromatographic behavior of the different types of enriched (modified) peptides shows that indeed the azacyclooctyne modified peptides elute at lower acetonitrile concentrations compared to the other two cyclooctyne moieties tested. The solubility of the modified peptides is increased and thereby the loss of material due to hydrophobic interactions is limited. In addition, it was observed that more ions were selected for fragmentation.

However, hardly any peptides were identified using Mascot for data analysis. Inspection of the fragmentation spectra learned that for most selected peptides

![Figure 4](image)

**Figure 4.** Fragmentation of linker of enriched products hampers identification of labeled peptides in complex mixtures. Loss of HF (or H\textsubscript{2}O) from the linker of peptides enriched by the F-ARCO-resin during fragmentation gives rise to a mother ion and two series of daughter ions. This lowers the score in Mascot searches and less protein identifications are obtained. Peptides enriched by the DIMAC-resin are modified by the azacyclooctyne, which upon fragmentation shows dominant breakage of the tertiary amine, hampering identification of proteins. a, Fragmentation spectrum of a peptide from azhal labeled Photoactive Yellow Protein (PYP) enriched by the F-ARCO-resin. TFDYQXTPTK at \textit{m/z} 1604.79. b, Overview of observed and assigned fragments in peptide sequence. y*: loss of HF. y*: loss of the cyclooctyne with the linker. ▲: cyclooctyne with linker with \textit{m/z} 420.2. ▼: linker after loss of HF with \textit{m/z} 400.2. ■: further fragmentation of the cyclooctyne gives rise to a benzoylium ion with \textit{m/z} 266.1. c, Fragmentation spectrum of a peptide belonging to pyruvate dehydrogenase enriched by the DIMAC-resin from a labeled \textit{E. coli} proteome is shown. With an ion score of 10, it was not assigned by Mascot. TFGXEGLFR at \textit{m/z} 1436.53. d, Overview of observed and assigned fragments in peptide sequence. y*: loss of the linker. ■: linker with \textit{m/z} 217.1. ▲: further fragmentation gives rise to a cyclization product with \textit{m/z} 117.0.
only little peptide backbone fragmentation took place. The loss of the linker was observed as a dominant process, as depicted in Figure 4. It can be best explained that the tertiary amide present in this group has a high proton affinity and is prone to fragmentation, as observed for tertiary amides present in proline residues. This resulted in low scores in the Mascot data analysis and most of the peptides selected for fragmentation have a score under the threshold for reliable identification. Therefore the DIMAC-resin was not used for further study.

This leaves us with the last cyclooctyne derivatized resin tested, the ARCO resin. The enrichment of a digest of azhal labeled PYP by the ARCO-resin allowed identification of all six azhal residues. Enrichment of peptides derived from a digest of a labeled *E. coli* proteome the ARCO-resin allows identification of more than twice as many peptides compared to the enrichment with the DIMAC-resin (100 peptides versus 40 peptides). The selective capturing and release of azide-containing peptides from different origin have been optimized for this resin and is described in great detail in Chapter 4. The resin has been successfully applied to study protein structures by chemical cross-linking (Chapter 5) and to study protein synthesis in *E. coli* by pulse labeling of newly synthesized protein by azhal (preliminary results are described in Chapter 6).

**Conclusion**

In this chapter it is shown that the requirements for an enrichment method for peptides stated in the introduction have been found to be valuable. First, reaction conditions during capturing or release of the peptides of interest should be compatible with mass spectrometric analysis, a criterion that could not be met for the PS-PEG resin, releasing mass spectrometric interfering molecules upon cleavage of the linker. Second, reaction conditions should be compatible with the enrichment, which was found not to be for the CPG beads, being destroyed by its reactive group, the fluorinated cyclooctyne. Third, the enrichment of peptides should achieve single products, ensuring correct identification of the peptides by data analysis. Both the Cu(I) catalyzed click reaction and the fluorinated cyclooctyne yielded unexpected modifications of target molecules, making identification difficult. And fourth, the last problem encountered was the unexpected fragmentation of the enriched products, making it impossible to correctly identify peptides enriched from more complex samples. This has shown that in the design of an enrichment method the fragmentation behavior of the isolated products is of great importance as well. The development of a covalent enrichment method makes it clear there are several hurdles that have
to be taken before a method is working properly. And even if a (nearly) perfect method has been found there are still further improvements that can be made, as will be discussed in the General Discussion of this thesis.

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Material and methods

Synthesis of cyclooctyne probes

The cyclooctyne moiety 4-(cyclooct-2-ynylmethyl)benzoic acid was synthesized as described by Agard et al.14 The protocol for the workup of the cyclooctyne methyl ester was adjusted, doubling the yield of this reaction step. Instead of using water, the reaction mixture was quenched with saturated NH₄Cl, extracted with EtOAc and dried over MgSO₄. After column chromatography (silica gel, 9:1 hexanes:EtOAc), the product was isolated as a colorless oil (50%). The other cyclooctyne probes were kindly provided by the group of Prof. C.R. Bertozzi; the fluorinated cyclooctyne by Dr. J. M. Baskin15 and the azacyclooctyne, 6,7-dimethoxyazacyclooct-4-yn (DIMAC) by Mrs. E. M. Sletten.22

Synthesis towards azide reactive Polystyrene-polyethyleneglycol (PS-PEG) resin

1 g of the PS-PEG resin Hypogel RAM 200 (0.6 mmol/g (Rapp Polymer, Tübingen, Germany) was washed with 12 mL DMF for 1 hour. The resin was treated with 5 mL 20% piperidine in DMF for 30 minutes. Deprotection of the amine of the RAM-linker was confirmed by the Kaiser test after which the resin was washed twice with 5 mL DF and CH₂Cl₂. Deprotected resin (1 eq or 1.2 eq) was reacted with a solution of HATU (O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate) (3 eq or 1 eq), Fmoc-4-Amb-OH (3 eq or 1 eq; ChemImpex, Illinois, USA) and diisopropyl-ethyl-amine (DIPEA) (6 eq or 2 eq) in dry DMF for 1.5 hours at room temperature. After a negative Kaiser test, the resin was washed with 10 mL DMF, 10 mL CH₂Cl₂ and 10 mL DMF for 15 minutes each and dried under nitrogen. The loading was determined as follows (ref: see below): 10 mg of resin was treated with 1 mL 20% piperidine in DMF for 10-15 minutes at room temperature, 50 times diluted with methanol, after which absorption was measured at 300 nm. The loading was determined to be 0.46 mmol/g (92%) and 0.22 mmol/g (50%) respectively.

Synthesis of azide reactive Controlled Pore Glass (CPG) beads

Aminopropyl CPG beads (0.2 mmol/g (200-400 mesh; Fluka, Missouri, USA) were derivatized with PAL linker, after which the loading was determined to be 0.19 mmol/g (yield: 95-106%). The cyclooctyne was coupled to the PAL linker, by incubating the derivatized resin (1 eq) with a solution of HATU (O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate) (3 eq), F-cyclooctyne (3 eq) and diisopropyl-ethyl-amine (DIPEA) (6 eq) in dry DMF for 1.5 hours at room temperature. Final loading of the fluorinated cyclooctyne was 0.17 mmol/g.
Synthesis of azide reactive PL-DMA resin

Azide reactive cyclooctyne (ARCO)-resin: 250 mg of PL-DMA resin (1.0 mmol/g 55-250µm (Polymer Laboratories, Shropshire, United Kingdom) was washed with 10 mL DMF, 10 mL CH₂Cl₂ and 10 mL DMF for 15 minutes each, prior use. The resin was reacted for three days with 15 mL of a 1 M cystamine solution (1 eq cystamine·2HCl, 2.5 eq DBU in DMF) in a reaction vessel at 30 °C under nitrogen. Positive coupling of cystamine to the PL-DMA beads was determined by bromo-phenol blue. After removal of the excess cystamine, the resin was washed by 10 mL DMF, 10 mL CH₂Cl₂, 10 mL DMF and 10 mL CH₂Cl₂ for 15 minutes respectively. The linker-derivatized resin (1.2 eq) was then reacted with a solution of HATU (O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate) (1 eq), cyclooctyne carboxylic acid (1 eq) and diisopropyl-ethyl-amine (DIPEA) (2 eq) in dry DMF for 3 hours at room temperature. After a negative bromo-phenol blue test, the resin was washed as described before and dried under nitrogen. To determine the loading of the cyclooctyne derivatized resin, 50 mg of resin was incubated with 250 mM Fmoc-Azhal in DMF for 24 hours at room temperature in a reaction vessel under nitrogen. Under these conditions it is expected that all cyclooctyne moieties are modified by the Fmoc-Azhal. The resin was washed and dried as described before. Then 2 mg of resin was incubated with 200 µL 20% piperidine in DMF for 15 minutes at room temperature (in duplo). The reaction mixture was diluted to 10 mL with MeOH and absorbance was recorded at 300 nm. Loading was calculated using the Lambert-Beer law, capacity (mmol/g) = (6.41*A300)/weight of the resin (mg). This yielded the ARCO-resin with a cyclooctyne loading of 0.25 mmol/g.

Fluorinated-azide reactive cyclooctyne (F-ARCO)-resin: as described for ARCO-resin, starting with 20 mg PL-DMA resin. The final loading was determined as described for the ARCO-resin to be 0.15 mmol/g.

DIMAC-resin: as described for ARCO-resin, starting with 90 mg PL-DMA resin. The final loading was not determined.

Preparation of peptide samples for enrichment

Two model peptides were used, each containing a single azidohomoalanine (X) residue: Pan016, an 18 amino acid peptide with the sequence PPHHHHHHPPRGFGXGFR and Pbn007, a seven amino acid peptide with the sequence FRFXGFFR. Photoactive Yellow Protein was produced as described in Back et al. Isolated protein was subjected to overnight digestion at 37 °C using a 1:50 (w/w) protease:protein ratio, with TPCK treated trypsin (Sigma-Aldrich, St. Louis, USA) in 50 mM potassium phosphate buffer pH 7.5. After digestion the peptide mixture was used for capturing experiments without any purification. A tryptic digest of an azhal labeled E. coli proteome was prepared as described by Kramer et al. Deviations from this protocol are described below. After washing, cells were transferred to M9 minimal medium in which methionine was replaced by 400 mg/L azhal and cells were grown for 2 hours in a shake flask at 37 °C. After tryptic digestion, reducing and alkylating reagents were removed with C18 tips, eluting in 75% acetonitrile.

Reactivity of alkyne probes towards an azidohomoalanine-containing model peptide

To a solution of model peptide Pan016 (2 nmol) and 2-ethynyl-pyridine (20 nmol) in 10 mM potassium phosphate buffer pH 8.0 were added CuSO₄ and ascorbate to a final concentration of 10 µM and 1 mM respectively (final volume 100 ul). Samples of 10 µl were taken before addition of reagents (t = 0) and after 10, 30 and 60 minutes of incubation at room temperature. Reagents were removed by desalting the sample on C18 reversed phase tips.
To model peptide Pan016 (1 nmol) in a solution of 25 mM potassium phosphate buffer pH 7.5 in 50% acetonitrile 1 μmol cyclooctyne moiety in DMF (final concentration 10% DMF) was added and incubated at 40 °C. Samples were taken at different time points, diluted with 0.1% TFA to a concentration of less than 5% ACN and desalted on C18 reversed phase tips.

**Enrichment procedure CPG beads**

Model peptide Pan016 and Pbn007 (3 nmol, 50 μL) in a solution of either 50 mM potassium phosphate buffer pH 7.5 or 25 mM potassium phosphate buffer pH 7.5 in 50% acetonitrile, was added to 3-4 mg dry beads and incubated at room temperature for 16 hours, under constant mixing. The beads were washed with a solution of 25 mM potassium phosphate buffer pH 7.5 in 50% acetonitrile, followed by washes of 15 minutes with acetonitrile; 25 mM potassium phosphate buffer pH 7.5 in 50% acetonitrile; 10 mM TEATFA pH 10.7; 3 M KCl in 10 mM TEATFA pH 10.7; 10 mM potassium phosphate buffer pH 7.5; 3 M KCl in 10 mM potassium phosphate buffer pH 7.5; 50% acetonitrile; acetonitrile; 50% acetonitrile; 10 mM sodium acetate pH 5.0 and water. For cleavage of the PAL linker, the beads were incubated with 95% TFA for 1 hour, 3 hours and 16 hours. After dilution of the 95% TFA with 1% formic acid, the sample was desalted on C18 reversed phase tips.

For Pan016, 50 ul of the reaction mixture of the enrichment in 25 mM phosphate buffer pH 7.5 in 50% acetonitrile was desalted on C18 reversed phase tips, dried for 5 minutes in the vacuum centrifuge and incubated with 95% TFA at room temperature for 16 hours. TFA was evaporated under vacuum and the dried sample was reconstituted in 4 ul of 60% acetonitrile 1% FA. For Pbn007, this procedure was employed for 5 ul of the cleavage product after 3 hours incubation with 95% TFA of the enrichment in 50 mM phosphate buffer pH 7.5.

**Enrichment procedure PL-DMA resin**

Model peptide Pan016 (~6 μg (3 nmol), 75 μL) and digests (AzPYP, 50 μg (~3 nmol), 75 μL; *E. coli* proteome, 400 μg, 150 μL) in a solution of 50% acetonitrile and 50% 50 mM potassium phosphate buffer pH 7.5, were added to dry beads. The mixtures were incubated with ~2 mg resin (Pan016 & AzPYP) or ~5 mg resin (*E. coli* proteome) at 40 °C for 24 hours, under constant mixing. To collect the supernatant the mixture was centrifuged. The beads were washed twice with a solution of 50% acetonitrile and 50% 50 mM potassium phosphate buffer pH 7.5, followed by washes of 15 minutes with acetonitrile; 50% acetonitrile, 50% 50 mM potassium phosphate buffer pH 7.5; 50 mM potassium phosphate buffer pH 7.5; 2 M NaCl and 50 mM potassium phosphate buffer pH 7.5. For cleavage of the disulfide linker, the beads were incubated with 5 mM TCEP in 50 mM potassium phosphate buffer pH 7.5 for 1 hour at room temperature, followed by 2.5 mM TCEP in a solution of 50% acetonitrile and 50% 50 mM potassium phosphate buffer pH 7.5 for 15 minutes. The two fractions were combined and iodoacetamide (55 mM final concentration) was added for 30 minutes at room temperature in the dark. Reducing and alkylating reagents were removed by desalting the peptide mixture on C18 reversed phase tips.

**Strong cation exchange chromatography of enriched *Escherichia coli* proteome samples**

For the enriched peptide mixtures by the ARCO- and DIMAC-resin all recovered material was loaded. Peptide mixtures were separated on a PolySULPHOETHYL Aspartamide™ Column (2.1mm ID, 10 cm length; PolyLC Inc., Columbia, USA) on an Ultimate HPLC system equipped with a Probot fraction collector (LC Packings, Amsterdam, The Netherlands). Elution (flow rate: 0.1 mL/min) was performed using a gradient of 30 minutes, from 0 to 250 mM KCl over the first 22.5 minutes, ending at 500 mM KCl after 30 minutes, with 20 mM potassium phosphate buffer pH 2.9, 20% acetonitrile as
buffer. 30 fractions of 100 μL were collected and lyophilized; fractions showing absorbance at 214 nm were redissolved in 0.1% TFA, 5% acetonitrile and used without any further purification for LC-ESI-Q-TOF analysis as described below.

**Mass spectrometry**

For reflectron MALDI-TOF, 0.5 μL sample was mixed with 0.5 μL of a 10 mg/mL α-hydroxy cinnamic acid solution in 1:1 (v:v) acetonitrile:ethanol and subsequently spotted on a MALDI target plate and dried. Spectra were recorded on a Tofspec 2EC mass spectrometer (Micromass, Wythenshawe, UK) provided with a 2 GHz digitizer.

Electrospray MS and low energy collision induced dissociation (MS/MS) analyses were performed on a Q-TOF (Micromass, Wythenshawe, UK) mass spectrometer with a Z-Spray orthogonal ESI source. For LC prior to MS, peptide mixtures were loaded on an Ultimate nano-HPLC system (LC Packings, Amsterdam, The Netherlands) and separated on a PepMap100 C\(_{18}\) reversed phase column (75 μm I.D., 25 cm length; Dionex, California, USA). For untreated samples ~10 pmol of material was loaded, for enriched AzPYP samples ~5% of the enriched material was injected. For each of the SCX fractions of the proteome sample before enrichment (100 ul) an amount corresponding to 10-15 mAU absorption at 214 nm was loaded. SCX fractions of the enriched *E. coli* proteome samples were split into two and run with both a linear (gradient B) and a step gradient (gradient C; see below). After loading on a trap column (Acclaim PepMap100, 300 μm I.D., 5 mm length, Dionex, Sunnyvale, CA, USA), elution (flow rate: 0.3 μl/min) of the peptides was performed using a linear gradient (gradient B), using solvent A, 0.1% formic acid and solvent B, 50% acetonitrile with 0.1% formic acid, going from 0% to 15% B over the first 12 min, then continuing to 45% B from 12-35 min and ending at 100% B at 45 min (gradient returned back to 0% B in 2 minutes). The step-gradient (gradient C) combined a linear and a step gradient, using solvent A, 0.1% formic acid and solvent C, 95% acetonitrile with 0.1% formic acid. After loading the following gradient was used; 0-5 min from 0 to 16% C, 5-8 min from 16 to 19% C, 8-10 min 19% C, 10.2-12.2 min 22% C, 12.4-14.4 min 24% C, 14.6-16.6 min 26% C, 16.8-18.8 min 28% C, 19-21 min 31% C, 21-27 min from 31 to 100% C, 27-37 min 100% C, and 37-40 min from 100% C to 0% C. Direct infusion of the flow was supported by a Nanobore Emitter (Proxeon, Odense, Denmark). Survey scans were acquired from m/z 350–1,750. For MS/MS most intense ions were selected in a data-dependent mode, recorded from m/z 50-2,500, scan time 1.00 sec, interscan delay 0.10 sec with argon as collision gas at a pressure of 4×10\(^{-5}\) bar measured on the quadrupole pressure gauge. Fragmentation spectra were processed with the MaxEnt3 algorithm included in the Masslynx Proteinlynx software, generating pkl-files (peak list).

**Data analysis**

Generated peak lists were submitted to Mascot (version 2.1, Matrix Science, London, UK) using an in-house license. Fragmentation spectra from AzPYP digests were searched against a database to which the sequence of the PYP construct was added. Analysis was carried out using following settings: TrypChymo, cleaving after K, R, Y, W, F and L except when a P follows, allowing up to 6 missed cleavages. No fixed modifications of cysteine residues were set, since the only cysteine residue in PYP is conjugated with the chromophore. Variable modifications included carbamidomethylation of cysteine; methionine replaced by azhal (C\(_{24}\)H\(_{31}\)FN\(_{6}\)O\(_{3}\), 502.216238) and H0-AZHAL (ARCO: C\(_{24}\)H\(_{32}\)N\(_{6}\)O\(_{4}\)S, 512.229128). Peptide tolerance was set at 0.5 Da, MS/MS tolerance at 0.5 Da, peptide charge at 1+. 41
Assigned fragmentation spectra were validated manually.

For *E. coli* data, fragment ion searches were carried out using a local database of the *E. coli* K12 proteome (4506 sequences; 1426768 residues, release 14.4 04/11/2008, Uniprot consortium, http://beta.uniprot.org/). Settings were as follows: Trypsin, allowing up to 1 missed cleavage; carbamidomethylation of cysteine residues as a fixed modification, variable modifications of methionine as described for AzPYP. Peptide tolerance was set at 0.8 Da, MS/MS tolerance at 0.8 Da, peptide charge at 1+. To estimate false positive protein identification rates (FPRs), fragment ion searches were performed against a decoy database, made using the Peakhardt decoy database builder (Medizinisches Proteom Center, Bochum, Germany; http://www.medizinisches-proteom-center.de), generating a shuffled version of the *E. coli* K12 proteome. FPRs were obtained by dividing the total number of protein hits from the decoy database by the total number of protein hits from the *E. coli* K12 database and multiplying this ratio by 100 percent. The Mascot output was filtered by using: a significance threshold of $p < 0.05$, MudPiT scoring on, ion score cutoff of 25 and selection of “require bold red”. For these search settings FPRs of less than 1.1% were obtained. Peptide summary reports were exported as csv-files (comma separated value) for further data-analysis in Excel (Microsoft Corporation, Redmond, USA). Exported Mascot results were filtered using the ASAP utilities add-on for Microsoft Excel (A Must in Every Office BV, Zwolle, the Netherlands; http://www.asap-utilities.com) for modified azhal peptides. Per experiment two files were generated containing proteins identified by either all unique peptides or by unique (modified) azhal-containing peptides. To calculate labeling numbers and enrichment percentages, methionine and azhal-containing peptides with the same primary sequence were counted separately.

References


Covalent solid phase enrichment method

27. A tryptic digest of an *E. coli* proteome yields around 25% of methionine-containing peptides. After pulse labeling to study protein synthesis, 2 to 10% of the proteins will contain the amino acid azhal instead of methionine, resulting in a peptide mixture of which 0.5 to 2.5% of the peptides contain an azide-group. From 5 mg of proteome 25 microgram of azhal-peptides is expected, corresponding to approximately 20 nmol of peptides.