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Protein Iodination by Click Chemistry

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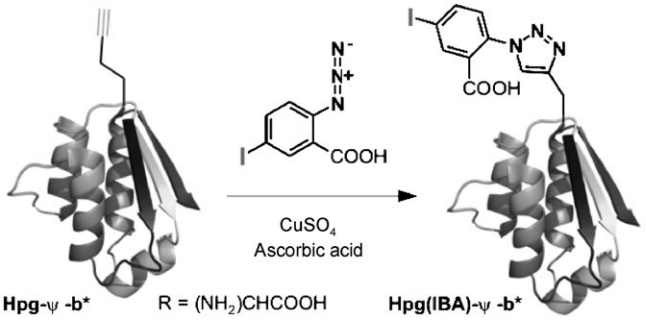
Upon development of radioimmunoassay techniques,[1] radioiodination of peptides and proteins has gradually become an indispensable tool for controlling and monitoring protein and peptide functions in vivo and in vitro, and for studying ligand–receptor interactions, ligand uptake and clearance. In addition, protein iodination has developed into a useful technique for heavy-metal modification in crystallographic analysis,[2] and serves as a valuable alternative to the expression of proteins with chalcogen amino acid analogues, such as selenomethionine, telluromethionine and selenocysteine.[3] Direct iodination of peptides and proteins at histidine, and more efficiently at tyrosine residues, under the various oxidative conditions is accompanied by modification of sensitive amino acid residues, such as methionine, cysteine and particularly tryptophan.[4] Indirect iodination methods that are based mainly on conjugation of peptides and folded proteins at surface exposed amine functions with iodinated *N*-hydroxysuccinimidyl-3-(4-hydroxyphenyl)propionate (Bolton–Hunter reagent[5]), and more recently with *N*-succinimidyl 3-iodobenzoate, have, therefore, been proposed.[6]

By using advanced procedures for expression of proteins with site-specific or residue-specific replacement of canonical amino acids with unnatural amino acids, iodophenylalanine has been introduced into proteins for solving the phase problem in X-ray crystallography,[2b] and access to suitable precursors containing reactive amino acid analogues for postexpression bioorthogonal modifications has been created.[7] Among the chemistries developed for such regio- and chemoselective modifications the Cu^{I}-catalyzed azide–alkyne Huisgen cycloaddition (CuAAC) reaction[8,9] has found widespread application. This procedure foresees the production of peptides and proteins with site- or residue-specific incorporation of alkyne- or azide-containing amino acid analogues by synthesis, semisynthesis or with suitable expression systems, which can then be treated with the complementary azide- or alkyne-containing label, respectively. In the case of synthetic peptides and smaller proteins, such functionalized residues can be incorporated at any sequence position, but care must be taken not to affect binding affinities to receptor molecules with the sequence modification or cause other related changes in conformational preferences. Consequently, for most proteins, N-terminal modification represents the method of choice, particularly in view of the full retention of structure and interacting epitopes.

As model protein, the engineered cysteine-free "pseudo-wild-type barstar" (ψ-b*; Pro28Ala/Cys41Ala/Cys83Ala), which consists of 90 amino acids and only one Met residue at the N terminus (Met1), was employed (Scheme 1). For successful



Scheme 1. Three-dimensional structure (ribbon plot; PDB entry: 1a19) of pseudo-wild-type barstar (ψ-b*: Pro28Ala/Cys41Ala/Cys83Ala).[11] The ψ-b* contains a single N-terminal Met, which was replaced with homopropargylglycine (Hpg) by using the auxotrophy-based residue-specific method, to give Hpg-ψ-b*. Iodination of Hpg-ψ-b* was achieved by using click chemistry in the presence of 2-azido-5-iodobenzoic acid (Scheme 2). The resulting 5-iodo-2-(4-alkyl-1*H*-1,2,3-triazol-1-yl)benzoic acid–protein conjugate is denoted as Hpg(IBA)-ψ-b*.

Met→Hpg replacement, recombinant ψ-b* containing *Escherichia coli* B834(DE3) host cells were grown in new minimal medium (NMM) containing Met (0.025 mM), until the amino acid was exhausted; this was followed by the simultaneous addition of Hpg and target-gene induction with IPTG (see the Supporting Information). Under these conditions, alkyne-labeled protein Hpg-ψ-b* was expressed in yields of ~80% compared to the native protein, that is, 8 mg L^{-1} versus 10 mg L^{-1}. Electrospray mass spectrometric analysis (ESI-MS) of Hpg-ψ-b* clearly revealed high replacement levels (approximately 90–95%) with low amounts of the parent protein as contaminant (Figure 1). In addition, the thermal stability of Hpg-ψ-b* (T_m = 68 °C) was nearly the same as that of the native protein (T_m = 67.9 °C[10]); this indicates that there was no significant effect on the protein structure upon Met→Hpg replacement.

Since the aim of the present study was the regioselective incorporation of radioactive iodide into a preselected position of a protein, it was desirable to have a precursor molecule that is readily iodinated and then transformed into the azide by chemistry tolerant of other functional groups for the subsequent direct reaction with the alkyne-containing protein in a largely aqueous solution.

Among the various precursors possibly suited for the synthesis of iodoaryl azides,[12] conversion of 4-iodophenyl boronic

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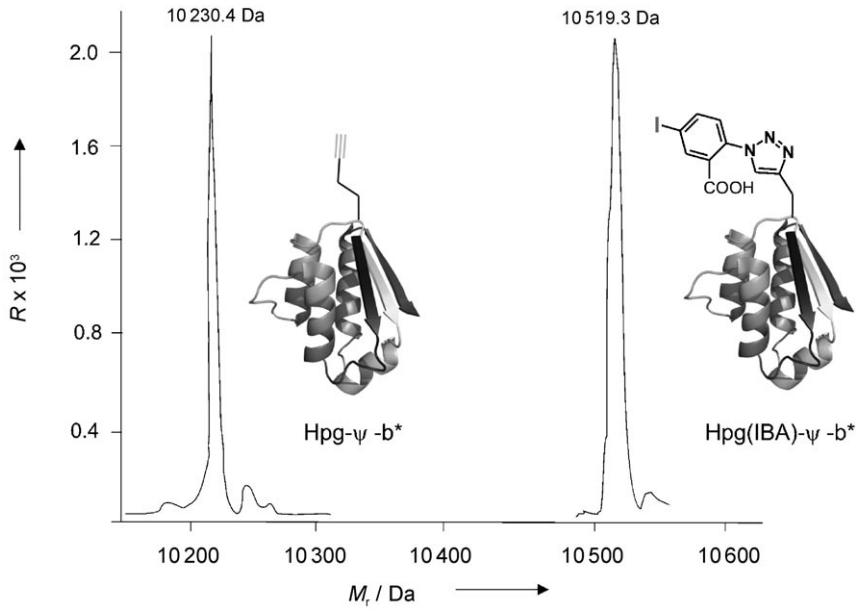


Figure 1. Deconvoluted ESI mass spectra profiles of Hpg-ψ-b* (found: 10230.4 Da; calcd: 10230.65 Da) and Hpg-(IBA)-ψ-b* (found: 10519.3 Da; calcd: 10519.69 Da), which was obtained after modification with 2-azido-5-iodobenzoic acid. Abbreviations: RI, relative intensity; M_r, relative molecular mass.

acid into 4-iodophenyl azide by treatment with sodium azide[12e] clearly showed insufficient solubility in aqueous solution in the presence of organic cosolvent for click chemistry with the chosen model protein. This led us to the choice of 2-amino-5-iodobenzoic acid, which is readily prepared from 2-aminobenzoic acid by iodination with ICl pyridine complex,[13] and then converted to the azide with an over 90% yield by treatment with *tert*-butyl nitrite in the presence of sodium azide, according to literature-known protocols[12b] (Scheme 2). As reported in the Experimental Section, aliquots of the azide mixture served for treatment with the Hpg-ψ-b* variant in PBS buffer (pH 8.0) at room temperature in the presence of a large excess of $CuSO_4$/ascorbic acid. LC-MS analysis of the reaction mixture revealed an almost quantitative conversion to the iodo-derivative (>95%) with traces of the parent protein as contaminant (Figure 1). The same chemistry was successfully performed with 5-azidofluorescein (see the Supporting Information).

As mentioned earlier, the Met→Hpg replacement at the N terminus of ψ-b* did not affect its stability. However, its subsequent iodination by CuAAC led to a less stable protein in terms of T_m value, which was lower by 4 °C as revealed in thermal unfolding experiments (Figure 2). This iodine conjugation most probably accounts for the less steep unfolding profile of Hpg(IBA)-ψ-b*, which might be interpreted as loss of structure cooperativity. The far UV CD spectra further confirmed such considerations. Indeed, the spectra for both Hpg-ψ-b* and Hpg(IBA)-ψ-b* exhibited the typical pattern of α/β proteins with the two negative maxima at 222 and 208 nm (Figure 2); this suggests that at least within the limits of this spectroscopic technique the identical overall fold was retained. However, the dichroic intensities of both negative maxima decreased in the spectrum of Hpg-(IBA)-ψ-b*; this supports slight changes in the secondary structure. In contrast, by measuring fluorescence emission maxima of both proteins no significant changes, neither in the λ_{max} nor in the intensities, could be detected (see the Supporting Information). These data suggest an intact tertiary protein structure upon N-terminal modification. However, minor interferences of the rather bulky hydrophobic 5-iodo-2-(4-alkyl-1*H*-1,2,3-triazol-1-yl)benzoic acid group with surrounding ordered structure elements cannot be excluded, as similar effects on the CD spectrum were previously observed upon N-terminal incorporation of carbohydrate moieties.[10]

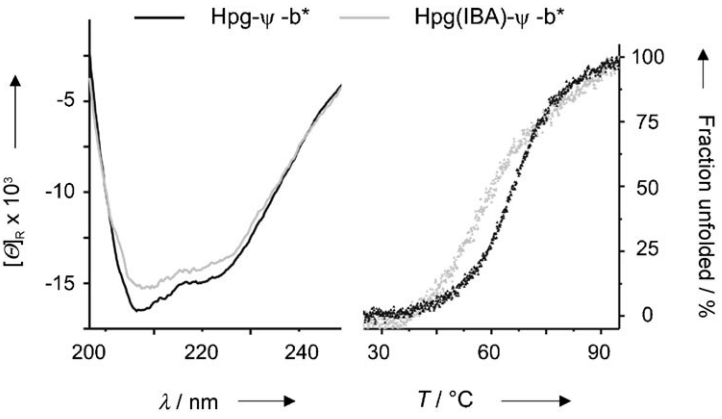
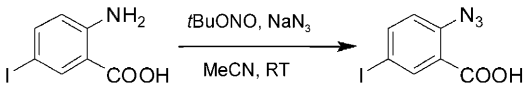


Figure 2. Secondary structures (left) and unfolding profiles (right) of Hpg-ψ-b* and Hpg-(IBA)-ψ-b*. Iodination of Hpg-ψ-b* with the triazole-linked 5-iodobenzoic acid attachment slightly destabilized the protein. The unfolding profile of Hpg(IBA)-ψ-b* was characterized by a negative shift of the T_m value (Hpg-ψ-b*: 68 °C; Hpg(IBA)-ψ-b*: 64 °C) and by decreased dichroic intensities. For experimental details see the Supporting Information. Dichroic intensities ($[\Theta]_R$) are expressed in deg cm^2 dmol^{-1}.



Scheme 2. Conversion of the iodoaryl amine into iodoaryl azide.

In view of these results, similar minor or major unfolding of protein secondary structures can also be expected with *N*-hydroxysuccinimidyl-3-(3-iodo-4-hydroxyphenyl)propionate or *N*-

succinimidyl-3-iodobenzoate reagents, depending on the various extents of protein derivatization at the amino groups. Compared to such conjugation chemistries, which exploit the rather abundant surface-exposed amine functions of proteins, the procedure proposed in the present communication should offer the advantage of a low number of Met residues that are generally surface-exposed in folded proteins. On the other hand, recombinant DNA technology has made it possible to introduce or eliminate Met residues at will, and subsequently many sequences with various positions for regioselective iodination can be prepared. The main advantage of this residue-specific modification over the expression of proteins with site-specific incorporation of iodinated amino acid residues in either cell-free[14] or in vivo systems,[2b] is the possibility to obtain larger amounts of precursor protein. In view of the half-life of the ^{125}I isotope, the protein can be (radio)iodinated just prior to its use. Moreover, the presence of the vicinal carboxy and adjacent 1,2,3-triazole groups should largely prevent extensive in vivo deiodination by deiodinases as observed for iodinated Tyr residues of proteins, or upon their modification with the Bolton–Hunter reagent (ref. [6b], and references therein).

Experimental Section

Solvents and reagents: All reagents were of the highest quality commercially available and were used without further purification. 2-Amino-5-iodobenzoic acid was purchased from Aldrich. LC-MS was performed by using a Bruker MicroTof and ESI-MS with a Perkin–Elmer SCIEX API 165 spectrometer equipped with a nebulizer-assisted electrospray. CD spectra were recorded with a Jasco 710 and fluorescence spectra were obtained with a Perkin–Elmer FL50B, at 20 °C. Protein samples (0.2 mg mL^{-1}) were diluted with 1×PBS buffer (pH 8). For CD measurements quartz Hellma 110-QS cells with optical path lengths of 0.1 cm were used, and the spectra were monitored at 50 nm min^{-1}, bandwidth 1 nm and response time 1 s; eight scans were accumulated in the 200–250 nm range. Fluorescence emission maxima were measured in the 300–450 nm range upon excitation at 280 nm at a protein concentration of 0.5 μM, which was obtained by diluting the reaction mixture before and after conjugation with 1×PBS buffer (pH 8.0).

2-Azido-5-iodobenzoic acid: Conversion of the amino group to azide was performed essentially by following literature procedures.[13] Briefly, 2-amino-5-iodobenzoic acid (142 mg, 0.54 mmol) was dissolved in acetonitrile (2 mL) and cooled to 0 °C in an ice bath. *tert*-Butylnitrite (120 μL, 1 mmol) was first added portion-wise to this solution while it was stirred, followed by NaN_3 (42 mg, 0.65 mmol). After 5 h at room temperature, conversion was found to be >90% as monitored by LC-MS.

Iodination reaction by CuAAC: An aliquot (4 μL) of the azide solution was added to 100 μL Hpg-ψ-b* (50 μM; 0.5 mg mL^{-1} in PBS, pH 8.0), followed by 8.0 μL $CuSO_4$ (25 mM in H_2O) and 8.0 μL L-ascorbic acid (50 mM in H_2O). The reaction mixture was shaken for 24 h at room temperature and then centrifuged. The extent of the reaction was assessed by LC-MS analysis.

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