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Notes & Tips

Role of arginine in chemical cross-linking with *N*-hydroxysuccinimide esters

Stefanie Mädler, Sabrina Gschwind, Renato Zenobi *

Department of Chemistry and Applied Biosciences, ETH Zurich, CH-8093 Zurich, Switzerland

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ABSTRACT

In order to clarify whether arginine has a promoting effect on the acylation of hydroxyl groups of serine, threonine, or tyrosine by homobifunctional cross-linking agents in aqueous solution, we carried out systematic experiments with model peptides, comparing relative reaction yields with covalently protected and unprotected arginines by MALDI-MS. The guanidinium group could be demonstrated to contribute to the reactivity of hydroxyl groups toward *N*-hydroxysuccinimide esters and catalyze the nucleophilic substitution, probably via hydrogen bonds.

Bioconjugation techniques are the basis for many innovative methods for the detection, characterization, and quantification of interactions between biomolecules. Numerous approaches rely on the highly amine reactive *N*-hydroxysuccinimide (NHS)[1] esters as specific and efficient immobilizing, labeling, or cross-linking agents. Apart from acylation of the ε-amino group of lysines and the α-amino group of N-termini, occasional side reactions with hydroxyl side chains have been observed [1–4]. Having pK_a values >13, the hydroxyl groups of serine (Ser) and threonine (Thr) are rather poor nucleophiles close to physiological pH. Although tyrosine (Tyr) is partly present as phenolate at pH values above or close to its pK_a = 9.8 [5], its reactivity toward NHS esters is still considerably lower than that of primary amines [4]. Additionally, the resulting ester bond has a somewhat lower stability than the amide bonds resulting from acylation of amino groups.

However, the presence of histidine (His) in sequences of the type His-Xxx-Tyr/Ser/Thr or His-Xxx-Xxx-Tyr/Ser/Thr (where Xxx = any amino acid) increases the reactivity of hydroxyl groups toward NHS labeling agents significantly [6]. Miller et al. have hypothesized that the imidazolyl group of His catalyzes the reaction via hydrogen bonding with the hydroxyl oxygen. For homobifunctional cross-linking agents, we have suggested a transient intramolecular link between His and the hydroxyl group as catalytic mechanism [4]. However, not all of the unusual reaction products with Ser or Thr detected in our recent work can be attributed to this kind of "histidine catalysis." Additional effects are likely to occur.

In protein chemistry, arginine (Arg), which is usually protonated under physiological conditions (pK_a = 12 [5]), often plays the role of a catalyst and thus fulfills numerous functions. As a Lewis acid, the guanidinium group can stabilize transition states with (partial) negative charges by means of hydrogen bonds and electrostratic interactions. In addition, proton transfer reactions to strong Brønsted bases are facilitated [7]. In order to clarify whether Arg has an enhancing effect on the acylation of hydroxyl groups by homobifunctional cross-linking agents in aqueous solution, we carried out systematic experiments. Peptides containing Ser, Thr, or Tyr in close proximity to Arg and control samples were selected.

Bradykinin (RPPGFSPFR, 1–9) and its fragments (1–8) and (2–9) (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) were chosen. Additionally, model peptides with the sequences DRLYSFGL-NH_2 (Allatostatin IV) and IGISNRDFV (Dengue 2 Virus Fragment) (both from AnaSpec, Inc., San Jose, CA), as well as NRCSQGSCWN (disulfide bridge), LQVQLSIR (Laminin α-1 Chain (2722–2729) (mouse)), VHFFKNIVTPRTP (Myelin Basic Protein (87–99)), and as negative controls Fmoc-EAALKLAR (Fmoc-(Glu^{70}, $Ala^{71,72}$, Lys^{74})-C3a and DPAFNSWG-NH_2 (Leucokinin I) (obtained from Bachem Holding AG, Bubendorf, Switzerland) were investigated. In order to be able to disregard other possible sequence-specific effects in comparative experiments, Arg residues were covalently blocked using a strategy proposed by Leitner and Lindner to map their solvent accessibility [8]. In a two-step cyclization reaction, the guanidinium group reacts selectively with 2,3-butanedione (BD) and phenylboronic acid (PBA) at pH 8–10 (Fig. 1).

Peptide solutions of 25 μM in 20 mM tetraethyl ammonium bicarbonate buffer (Sigma–Aldrich, pH 9.4) were either diluted with water for control experiments or incubated with 2,3-butanedione (Alfa Aesar GmbH & Co., Karlsruhe, Germany) and phenylboronic acid (Sigma–Aldrich) in a 200- and 400-fold excess per Arg, respectively, to reach a final peptide concentration of 12.5 μM. After 1 day of incubation time, a 100-fold molar excess of the NHS esters disuccinimidyl suberate (DSS, Pierce, Rockford, IL) or

* Corresponding author. Fax: +41 44 632 12 92.
E-mail address: zenobi@org.chem.ethz.ch (R. Zenobi).

[1] *Abbreviations used*: BD, 2,3-butanedione; BS3, bis(sulfosuccinimidyl) suberate; DSS, disuccinimidyl suberate; ESI-Q-TOF, electrospray ionization quadrupole time-of-flight; His, histidine; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer; NHS, *N*-hydroxysuccinimide; PBA, phenylboronic acid; Ser, serine; TFA, trifluoroacetic acid; Thr, threonine; Tyr, tyrosine.

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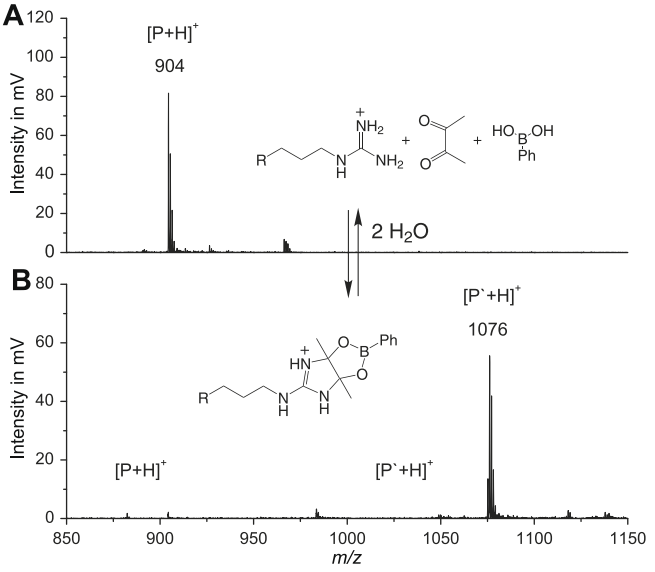

Fig. 1. Protection of Arg with 2,3-butadione and phenylboronic acid. The MALDI spectra of PPGFSPFR (A) before and (B) after protection show the completeness of the reaction of R8 indicated by a shift of 172 u between P and P′.

bis(sulfosuccinimidyl) suberate (BS^3, Pierce) dissolved in dimethylformamide was added to the solution (causing the pH to drop to about 8.1), and allowed to react for 3 days at room temperature to obtain maximum hydroxyl modification. To remove the acid-labile protection group and thus to ensure comparable ionization efficiencies, the solutions were acidified with trifluoroacetic acid (TFA) to reach a final acid concentration of 1% (v/v) followed by ZipTip purification (0.6 μL C18 resin, Millipore, Molsheim, France).

Reaction solutions were mixed with 10 mg/mL of matrix solution and 1 μL of the mixture was spotted onto a stainless-steel plate and allowed to dry under ambient conditions. As matrix, α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich) was dissolved in acetonitrile/water/TFA (49.95/49.95/0.1, v/v/v). For checking the degree of covalent modification of Arg with the acid-labile boronic acid ester, a nonacidic matrix, 2′,6′-dihydroxyacetophenone (Sigma–Aldrich) dissolved in methanol/water (50/50, v/v) was used.

For the analysis of the reaction products, a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) (Axima CFR, Kratos Analytics, Shimadzu Biotech, Manchester, UK) equipped with a nitrogen laser (λ = 337 nm) operating at 10 Hz was used. An UltraFlex II MALDI-TOF-MS (Bruker Daltonics GmbH, Bremen, Germany) equipped with a "smartbeam" laser with λ = 355 nm operating at 100 Hz was also used, particularly to estimate the reaction yields. Experiments were performed in reflectron mode and delayed extraction. One thousand scans obtained at different positions were accumulated for one spectrum, and 5 spectra were recorded for each sample and baseline subtracted.

The covalent protection of Arg results in a mass shift of 172 u. Before and after cross-linking, virtually complete protection of Arg residues was observed by MALDI-MS (Fig. 1).

In order to identify modified reaction sites, MS/MS measurements were performed in positive-ion mode either on an electrospray ionization quadrupole TOF (ESI-Q-TOF) MS (Q-ToF ULTIMA, Waters, Milford, USA) equipped with an automated chip-based nanoelectrospray ionization system (Nanomate 100, Advion Biosciences, Ithaka, USA) or on a 4800 MALDI TOF/TOF analyzer (Applied Biosystems AB, MDS SCIEX, Foster City, CA).

The two different modification types detected in our experiment will be named according to the nomenclature introduced by Schilling et al. [9]: the term "Type-0 cross-link" refers to the modification of a peptide (P) with a cross-linker (XL) whose other end is hydrolyzed, abbreviated herein as P-XL$_{OH}$. A "Type-1 cross-link" describes a modification where both reactive ends reacted within a single peptide (P = XL). After 3 days of cross-linking, only P = XL and P-XL$_{OH}$ were observed with MALDI-MS. As confirmed by MS/MS, P-XL$_{OH}$ species resulted primarily from the modification of the α-amino group of the N-terminus. Reactions of Ser, Thr, and Tyr occurred predominately as Type-1 linkages connecting the N-termini and the hydroxyl groups for all investigated peptides. Thus, the high local concentration of the cross-linker attached to the N-terminus and entropic effects favored the nucleophilic attack of hydroxyl groups, consistent with our previous findings [4].

With the intention of studying the possible increase in reactivity resulting from Arg, we performed cross-linking experiments with and without Arg being blocked. The protection group prevents any potential hydrogen bonds or reactions with the cross-linker. Based on the MALDI spectra, we compared the peak integrals of P = XL relative to the sum of P = XL and P-XL$_{OH}$ resulting in the ratio R(%) for the experiments with and without Arg protected. The R(%) values as well as its relative changes ΔR(%) are illustrated in Fig. 2. Interestingly, DRLYSFGL-NH$_2$, IGISNRDFV, PPGFSPFR, VHFFKNIVTPRTP, and NRCSQGSCWN showed a significant decrease of R(%), i.e., a lower reactivity of the hydroxyl groups when Arg was covalently blocked. Two control experiments were performed, (i) mixing BD and PBA with the Arg-free peptide DPAFNSWG-NH$_2$ and (ii) adding only PBA to PPGFSPFR. No changes of R(%) were observed, thus confirming the direct effect of Arg blockage. The peptides RPPGFSPFR and RPPGFSPF both have an N-terminal Arg. However, the experimentally confirmed blocking of R1 did not have any significant influence on the amount of Type-1 cross-

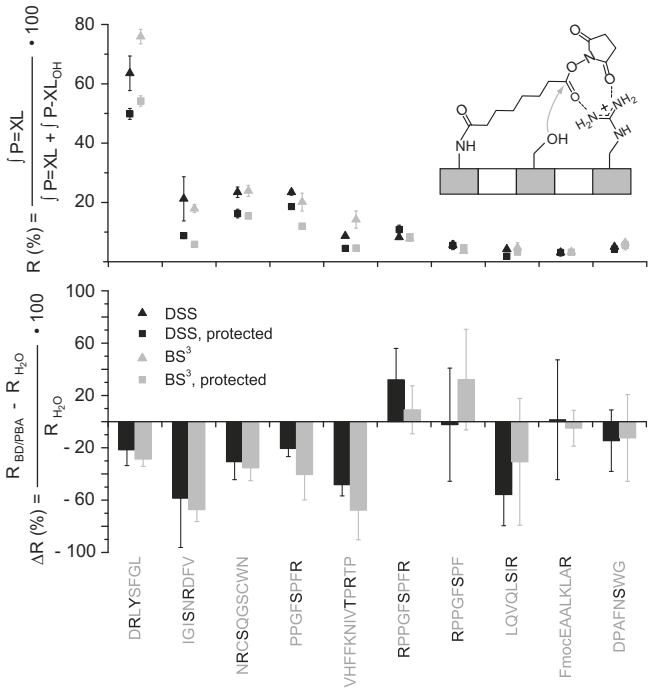

Fig. 2. Effects of a protected guanidinium group on the amount of hydroxyl acylation for the cross-linking of peptides with DSS or BS^3. Mainly reacting hydroxyl amino acids and Arg are indicated in black. Based on the MALDI spectra, two different values were calculated: (A) the relative amount of P = XL R(%) and (B) its relative changes ΔR(%). Error bars indicate one standard deviation. The 5 first peptides show a significant decrease of R(%) with a blocked Arg. The control peptides Fmoc-EAALKLAR and DPANSWG did not exhibit any Arg effect. The Arg effect is assumed to result from hydrogen bonds between the guanidinium group and the carbonyl oxygens of the cross-linker.

linked species in both cases. A steric effect of the rather voluminous boronic acid ester functionality is thus unlikely.

In general, the amount of P = XL depended strongly on the distance between the N-terminus and the corresponding S, T, or Y. As demonstrated in Fig. 2, *R*(%) tends to decrease with increasing bridging distance. For DRLYSFGL-NH_2, IGISNRDFV, PPGFSPFR, VHFFKNIVTPRTP, and NRCSQGSCWN, S, T, or Y are situated in positions 4 to 5 considering the closest primary amine as position 1. For the peptides with distances of 6 amino acids RPPGFSPFR, RPPGFSPF, and LQVQLSIR, only low amounts of P = XL were created and no significant intensity change was detected. Small peptides of this size occupy a large conformational space in aqueous solution, as demonstrated, for example, for RPPGFSPFR by Denys et al. [10]. Thus, the distance between the α-amino and the hydroxyl group should theoretically be well within reach of the spacer arm lengths of 11.4 Å. The low amount of P = XL and its invariant intensity are therefore probably a consequence of hydrolysis of the second cross-linker end rather than its reaction with a peptide hydroxyl group.

In order to elucidate the mechanism of the assumed Arg catalysis, we investigated if Arg forms stable products with DSS and BS^3. MALDI spectra of the peptide Fmoc-EAALKLAR revealed products with *m*/*z* corresponding to P-XL_{OH} (+156 u) and P = XL (+138 u) after cross-linking. The product ion spectra confirmed the labeling of K5 for P-XL_{OH}, but could not unambiguously prove the formation of P = XL between K5 and R8. As illustrated in Fig. 2, the protection of R8 did not result in a change of *R*(%) either. Instead of a nucleophilic reaction of Arg, the loss of water from the 156 u adduct to form the 138 u adduct during ionization is thus very likely. This finding is consistent with the results of Cuatrecasas and Parikh who did not find any interference of N-acetylated arginine with the reaction of N-terminal free alanine with immobilized NHS esters [11].

Thus, as an alternative mechanism, the formation of hydrogen bonds between the guanidinium hydrogens and the oxygen of the attacked carbonyl group of the cross-linker should be considered to facilitate the nucleophilic attack of the hydroxyl groups by lowering the activation barrier of the nucleophilic substitution (Fig. 2). The hydrolysis of the cross-linker could be promoted by the same mechanism, but seems to have a smaller effect on the P = XL intensity than the reduction of hydroxyl reactivity under the conditions used.

The sulfonate group of BS^3 also represents a good target for interaction with the guanidinium moiety, given that sulfonates have been successfully applied as noncovalent receptor to probe the surface accessibility of arginines [12]. However, since no significant differences of *R*(%) were observed between the two cross-linkers, the sulfonate–Arg interaction probably does not have a large influence on the reactivity of hydroxyl groups. In summary, Ser, Thr, and Tyr are of significant importance as reactive sites for chemical cross-linking and can be used as additional constraints for structure elucidation studies. The guanidinium group of Arg was demonstrated to contribute to the reactivity of hydroxyl groups toward NHS esters and to catalyze the nucleophilic substitution probably via hydrogen bonds. This finding can explain the enhanced reactivity of Ser, Thr, or Tyr in Arg-containing peptides.

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