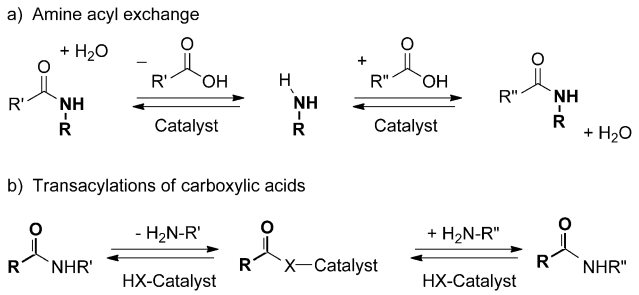
Enzyme Catalysis

DOI: 10.1002/anie.201205404

Enzymatic Amine Acyl Exchange in Peptides on Gold Surfaces**

*Roberto Castangia, Martina Austeri, and Sabine L. Flitsch**

Syntheses and reactions of amides catalyzed by enzymes are fundamental in biology, chemistry, biotechnology, and nanotechnology. Among the repertoire of transformations of amides, their syntheses from carboxylates, activated esters, and amides through transacylations of carboxylic acids have been widely studied (Scheme 1b). However, the amine acyl exchange of nonactivated amides with carboxylic acids using enzymes (Scheme 1a) has been little explored. An elegant chemical method using perfluorinated anhydrides described recently[1] requires activation both of the amide and carboxylic acid, is limited to secondary amides, and lacks chemoselectivity. More selective and biocompatible amine acyl exchange can be potentially provided using enzymes; this approach would not require activation of the carboxylate and in addition would be highly stereo- and chemoselective.



Scheme 1. Amine acyl exchange (a) versus transacylation (b).

Of the reported enzyme-catalyzed interconversions of amides, almost all can be classified as transacylations of carboxylates (Scheme 1b). Examples are transacylations catalyzed by transglutaminase,[2] sortase,[3] diverse proteases,[4] and lipases such as CalB.[5] There are some key mechanistic differences between the two reactions: Amine acyl exchange involves the reversible interconversion of amines and free carboxylic acids, whereas the transacylation of carboxylates relies on the formation and selective reaction of an acyl–enzyme intermediate. Importantly, hydrolysis can be avoided in the latter by kinetic control of the reaction, for example by redirection of the reaction pathway through the catalyst–acyl intermediate thus avoiding stable free carboxylates (Scheme 1b). The amine acyl exchange (Scheme 1a) is more challenging as it relies on equilibria and more stable carboxylates as substrates. Such an enzyme-catalyzed acyl exchange has, to our knowledge, been reported only for very limited amines such as urea.[6]

Transpeptidations have been described in biological systems as being responsible for the splicing of peptides and proteins in the proteasome, but have always been attributed to the reaction type in Scheme 1b.[7] It would be interesting to see if amine acyl exchange catalyzed by proteases, which do not rely on acyl–enzyme intermediates, are also feasible under closer-to-physiological reaction conditions and hence could contribute to peptide splicing. Such reactions would proceed through stable intermediates and have less stringent requirements on the temporal and spacial availability of reaction partners. We were therefore looking for a reaction system that allowed us to investigate enzyme-catalyzed amine acyl exchange in aqueous conditions.

We had previously described both the hydrolysis and synthesis of amides (the first and second step of the reaction in Scheme 1a) using proteases on substrates that are linked to solid support (R) through the amine partner.[8] In particular, functionalized self-assembled monolayers (SAMs) attached to gold surfaces have proven to be fully compatible both with chemical and biochemical reactions and have allowed us to investigate protease-catalyzed reactions. Any transformation can be conveniently studied observed in this system in a label-free manner using in situ MALDI-Tof mass spectrometry. Given our previous success with protease-catalyzed peptide synthesis on such surfaces,[9] it was decided to use the same platform to see if acyl exchange of amides could be observed.

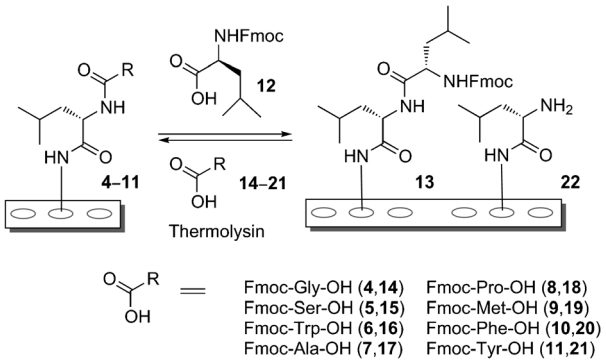
Accordingly, spot surfaces in a 64-well gold array (suitable for MALDI-Tof measurements) were incubated with DMSO solutions of functionalized alkane thiols to form an array of self-assembled monolayers.[10] The density of functional groups was controlled by using the functionalized alkane thiols [$HS(CH_2)_{17}(OCH_2CH_2)_6$ OCH_2COOH] (**1**) and [$HS(CH_2)_{17}(OCH_2CH_2)_3OH$] (**2**) in defined proportions. *N*-Fmoc-diaminobutane (**3**) was then coupled to **1** by *N*-succinimide activation and then the Fmoc group was cleaved. Using standard peptide coupling reactions, nine different Fmoc-protected dipeptides were coupled to this functionalized linker[9] generating dipeptide surfaces **4**–**11** (Scheme 2); this process was monitored by MALDI-Tof MS (see Figure 1a for data on compound **9**).

The surface-linked dipeptides **4**–**11** were then treated with the protease thermolysin from *B. thermoproteolyticus rocco* in the presence of excess Fmoc-leucine (**12**) (Scheme 2); **12** was chosen since leucine is known to be a good substrate for thermolysin in the P_1 position.[11] A wide range of reaction conditions were tested, and it was found that 4 M NaCl in Tris buffer (100 mM, pH 7.5) increased the yield of product.[12]

[*] R. Castangia, Dr. M. Austeri, Prof. S. L. Flitsch
The School of Chemistry and MIB, The University of Manchester
131 Princess Street, Manchester M1 7DN (UK)
E-mail: sabine.flitsch@manchester.ac.uk

[**] This project was funded by the EPSRC, European Union FP6&7, Swiss National Science Foundation (to M.A.), and Royal Society (Wolfson Merit award to S.L.F.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201205404.



Scheme 2. Thermolysin-catalyzed reversible amine acyl exchange on gold arrays.

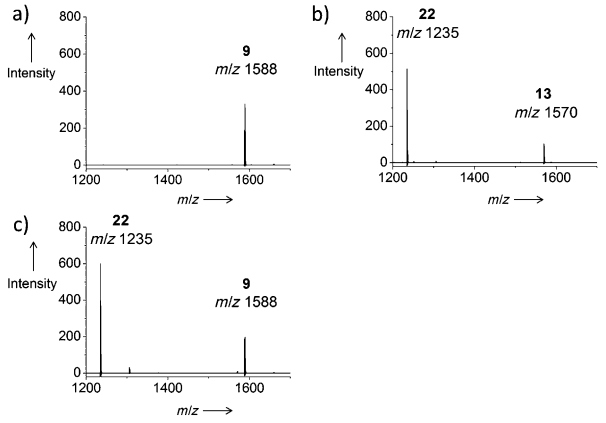


Figure 1. MALDI-Tof spectra recorded during acyl exchange of **9**. a) Starting material **9** (*m*/*z* 1588) as the disulfide dimer sodium adduct. b) After the first exchange with thermolysin and Fmoc-Leu, **9** appears to be totally converted to **13** (*m*/*z* 1570), and hydrolysis product **22** (*m*/*z* 1235). c) Second acyl exchange of the product from reaction (b) with Fmoc-Met converts **13** back to **9** (*m*/*z* 1588).

Figure 1 b shows the mass spectrum for conversion of **9** to **13** (*m*/*z* 1570).[13] It appears that **9** was fully converted to **13** and hydrolysis product **22** (*m*/*z* 1235). Similar results were obtained for dipeptides **4**–**7** and **9**–**11**. Only the proline derivative **8** resulted in a mixture of starting material **8** and product **13**, possibly because of the low activity of thermolysin towards prolines at the P_1 site, which is documented.[11b,c,14]

The reversibility of the amine acyl exchange was further investigated by treating all the surface-bound products of the above reactions with a fresh solution of protease, this time with different Fmoc-protected amino acids **14**–**21** instead of Fmoc-leucine (**12**) (i.e. reaction follows bottom reaction arrow in Scheme 2).

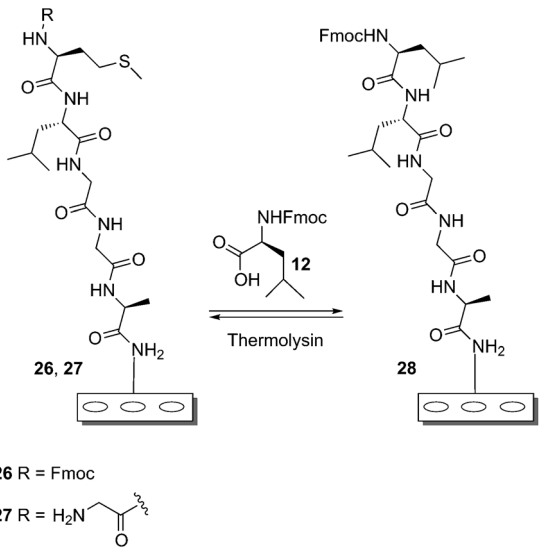
Figure 1 c shows the mass spectrum after treatment of the sample shown in Figure 1 b with excess Fmoc-methionine (**19**), generating a surface that appears to contain no starting material **13** (expected signal at *m*/*z* 1570) and contains **9** (*m*/*z* 1588) as the only detectable dipeptide. Similar results were obtained for treatment of **13** with Fmoc-alanine (**17**) and Fmoc-tyrosine (**21**), whereas Fmoc-serine (**15**), Fmoc-tryptophan (**16**), and Fmoc-proline (**18**) generated mixtures with **13**, possibly again owing to the substrate specificity of the enzyme. Performing the reaction with Fmoc-glycine (**14**) and Fmoc-phenylalanine (**20**) gave no products.

Given that there are a great number of different classes of proteases with different catalytic mechanisms, it was interesting to see whether the amine acyl exchange reaction was limited to thermolysin (a zinc-dependent protease) or whether it could also be observed with serine proteases such as α-chymotrypsin. Although the substrate specificity of α-chymotrypsin is different from that of thermolysin, some of the compounds shown in Scheme 2 should be substrates for both proteases and were therefore useful for direct comparative studies into the acyl exchange activity of both proteases. Thus the immobilized dipeptides Fmoc-Tyr-Leu-R^s (**11**, where R^s is the solid phase) and Fmoc-Tyr-Phe-R^s (**23**) were incubated with Fmoc-Leu (**12**) and Fmoc-Phe (**20**) yielding the products Fmoc-Leu-Leu-R^s (**13**), Fmoc-Phe-Leu-R^s (**10**), and Fmoc-Phe-Phe-R^s (**24**), respectively, as well as the hydrolysis products Leu-R^s (**22**) and Phe-R^s (**25**). These results would suggest that amine acyl exchange can be performed with different classes of proteases; the reaction is not protease-mechanism dependent and possibly proceeds under thermodynamic rather than kinetic control.

A great advantage of enzymatic over chemical methods is the potential for stereoselectivity, which cannot be achieved with current chemical methods for acyl exchange. Thus, we investigated the enzymatic transformations of enantiomers L,L-**9**, D,L-**9**, L,D-**9**, and D,D-**9** with thermolysin in the presence of isomers L-**12** and D-**12** under the reaction conditions outlined in Scheme 2. The results showed that the amine acyl exchange was indeed stereoselective—only the reaction between L,L-**9** and L-**12** yielded a product corresponding to the dipeptide product **13** (see the Supporting Information for full experimental data; Figures S11 and S12). These results also confirm that the acyl exchange reactions are indeed enzyme-catalyzed and not due to chemical background reactions.

Apart from stereoselectivity, one might also expect to observe chemoselectivity for protease-catalyzed acyl exchange reactions, since the reactions of proteases are generally linkage-specific. Thus, the enzyme should be able to exchange structurally different peptide bonds although they might have similar chemical reactivity. To probe such selectivity, two peptides **26** and **27** (Scheme 3) were synthesized directly on the gold array through spot synthesis.[15] Compound **26** is a pentapeptide with an N-terminal Fmoc group whereas hexapeptide **27** contains a free N-terminal glycine residue (for further examples of shorter peptides **31, 34, 37, 38**, and **39**, see the Supporting Information). When **26** and **27** were treated with thermolysin in the presence of Fmoc-leucine (**12**) as in Scheme 3, the resulting mass spectra clearly showed that only the Met-Leu linkage underwent exchange to give the peptide **28** in both cases with some hydrolysis side products. Thus, the enzyme selectively exchanges one out of four and five amide bonds in **26** and **27**, respectively.

In conclusion, we have developed reaction conditions in aqueous buffer which allow us to observe reversible protease-catalyzed amine acyl exchange reactions. The reactions proceed under mild biocompatible reactions conditions and



Scheme 3. Enzyme-catalyzed acyl exchange of peptides on gold arrays. (For a detailed experimental procedure, see the Supporting Information.)

are stereo- and chemoselective. We have shown that the amine acyl exchange can be catalyzed by different classes of proteases (the zinc-dependent protease thermolysin and the serine protease chymotrypsin). To our knowledge this is the first time that protease-catalyzed transpeptidations in aqueous media have been shown to proceed as amine acyl exchange and not transacylation. These exchange reactions should be useful for biocompatible and selective modifications of amine-functionalized surfaces in "bottom-up" bionanofabrication strategies.[16]

Our studies also suggest that amine acyl exchange reactions are possible under physiological conditions and should be considered as mechanisms for peptide splicing observed in the human proteasome.[7] Peptide splicing produces immunogenic peptides composed of two noncontiguous fragments from the parental proteins and may be relevant for vaccine design. These transpeptidations have in the past been attributed to transacylations of carboxylates (Scheme 1 b), but our results here suggest that proteases should also be able to catalyze amine acyl exchange reactions (Scheme 1 a) which are driven by equilibrium rather than kinetics and should have distinctly different mechanism and substrate requirements.

Received: July 9, 2012
Revised: August 30, 2012
Published online: November 20, 2012

Keywords: chymotrypsin · enzyme catalysis · peptides · thermolysin · transacylation

[1] a) P. Rota, P. Allevi, R. Colombo, M. L. Costa, M. Anastasia, *Angew. Chem.* **2010**, *122*, 1894–1897; *Angew. Chem. Int. Ed.* **2010**, *49*, 1850–1853; b) P. Rota, P. Allevi, I. S. Agnolin, R. Mattina, N. Papini, M. Anastasia, *Org. Biomol. Chem.* **2012**, *10*, 2885.
[2] L. Fornelli, A. W. Schmid, L. Grasso, H. Vogel, Y. O. Tsybin, *Chem. Eur. J.* **2011**, *17*, 486–497.
[3] K. Piotukh, B. Geltinger, N. Heinrich, F. Gerth, M. Beyermann, C. Freund, D. Schwarzer, *J. Am. Chem. Soc.* **2011**, *133*, 17536–17539.
[4] C. R. Berkers, A. de Jong, H. Ovaa, B. Rodenko, *Int. J. Biochem. Cell Biol.* **2009**, *41*, 66–71.
[5] a) O. Torre, E. Busto, V. Gotor-Fernandez, V. Gotor, *Adv. Synth. Catal.* **2007**, *349*, 1481–1488; b) P. O. Syrén, K. Hult, *ChemCatChem* **2011**, *3*, 853–860; c) G. Cardillo, A. Gennari, L. Gentilucci, E. Mosconi, A. Tolomelli, S. Troisi, *Tetrahedron: Asymmetry* **2010**, *21*, 96–102.
[6] N. P. Awasthi, R. P. Singh, *J. Oleo Sci.* **2007**, *56*, 507–509.
[7] a) A. Dalet, V. Stroobant, N. Vigneron, B. J. van den Eynde, *Eur. J. Immunol.* **2011**, *41*, 39; b) J. Liepe, M. Mishto, K. Textoris-Taube, K. Janek, C. Keller, P. Henklein, P. M. Kloetzel, A. Zaikin, *PLoS Comput. Biol.* **2010**, *6*, e1000830; c) A. Dalet, N. Vigneron, V. Stroobant, K. Hanada, B. J. Van den Eynde, *J. Immunol.* **2010**, *184*, 3016; d) N. Vigneron, V. Stroobant, J. Chapiro, A. Ooms, G. Degiovanni, S. Morel, P. van der Bruggen, T. Boon, B. J. Van den Eynde, *Science* **2004**, *304*, 587.
[8] R. V. Ulijn, B. Baragana, P. J. Halling, S. L. Flitsch, *J. Am. Chem. Soc.* **2002**, *124*, 10988–10989.
[9] R. Haddoub, M. Dauner, F. A. Stefanowicz, V. Barattini, N. Laurent, S. L. Flitsch, *Org. Biomol. Chem.* **2009**, *7*, 665–670.
[10] a) J. C. Love, L. A. Estroff, J. K. Kriebel, R. G. Nuzzo, G. M. Whitesides, *Chem. Rev.* **2005**, *105*, 1103–1169; b) B. T. Houseman, J. H. Huh, S. J. Kron, M. Mrksich, *Nat. Biotechnol.* **2002**, *20*, 270–274; c) N. Laurent, J. Voglmeir, A. Wright, J. Blackburn, N. T. Pham, S. C. C. Wong, S. J. Gaskell, S. L. Flitsch, *ChemBioChem* **2008**, *9*, 883.
[11] a) B. W. Matthews, *Acc. Chem. Res.* **1988**, *21*, 333–340; b) H. Matsubara, *Biochem. Biophys. Res. Commun.* **1966**, *24*, 427–430; c) H. Matsubara, R. Sasaki, A. Singer, T. H. Jukes, *Arch. Biochem. Biophys.* **1966**, *115*, 324–331.
[12] K. Inouye, *J. Biochem.* **1992**, *112*, 335–340.
[13] Typical experimental procedure: Fmoc-protected amino acid (40 mg mL^{-1}) **12** was dispersed in 100 mM Tris buffer (pH 7.5) containing 10 mM $CaCl_2$ and 4 M NaCl. Thermolysin (0.01 mg mL^{-1}) was then added. The mixture was spotted (0.5 μL per well) onto the funtionalized surface containing peptides **4**–**11**. The reactions were incubated at 37 °C for 30 min. The reactions were stopped washing the chip with a 0.1 % formic acid solution in water. Analysis was carried out by MALDI-ToF MS (Bruker Ultraflex II TOF/TOF). A 55 mM THAP (2,4,6-trihydroxyacetophenone monohydrate) matrix solution in acetone was used. To avoid the formation of potassium adducts, a 1 % solution of 10 μM NaCl in HPLC-grade water was added to the matrix solution. Spectra were acquired in reflectron positive mode with a nitrogen laser (337 nm) attenuation 40 %, ion source 25 kV. Spectra were analyzed with Flexanalysis Software (Bruker,USA) using default integration settings.
[14] R. L. Heinrikson, *Methods Enzymol.* **1977**, *47*, 175–189.
[15] N. Laurent, R. Haddoub, J. Voglmeir, S. C. C. Wong, S. J. Gaskell, S. L. Flitsch, *ChemBioChem* **2008**, *9*, 2592–2596.
[16] A. R. Hirst, B. Escuder, J. F. Miravet, D. K. Smith, *Angew. Chem.* **2008**, *120*, 8122–8139; *Angew. Chem. Int. Ed.* **2008**, *47*, 8002–8018.

