benzotriazol (HOAT) as activating reagents and the coupling times were extended to 3 – 4 h to avoid incomplete peptide bond formation.

After the entire biotin-tagged and farnesylated peptide **23** had been assembled on the polymeric support, the seven Aloc groups present were removed simultaneously by treatment with $Pd[PPh_3]_4$ in the presence of piperidine for four hours. Removal of the catalyst was achieved by simple washing, which rendered the troublesome purification of the unmasked oligolysine peptide unnecessary.

Finally, fully unmasked lipidated K-Ras peptide **2** was released from the solid support by treatment with 1% TFA in the presence of 2% TES. Under these conditions both *O*-trityl groups present in **24** were removed as well and the farnesyl group remained unattacked.[16] Purification of the target peptide was readily achieved by means of HPLC on an RP-C18 column to yield the desired biotin-tagged and lipidated oligolysine peptide **2** (Figure 1) in high purity and with 11% overall yield.

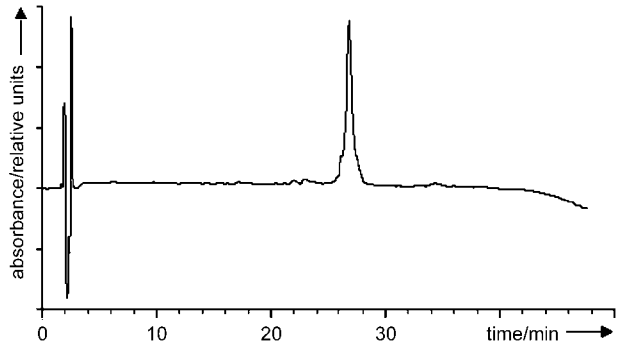

Figure 1. *HPLC analysis of lipidated polybasic Ras-peptide **2**. Detection at 210 nm; RP-C18PPN column; solvent A: water; solvent B: acetonitrile; solvent C: water with 1% TFA; gradient: 0 min 80% A, 10% B, 10% C; 40 min 35% A, 55% B, 10% C; retention time: 26.9 min.*

In summary, we have developed a reliable and practical method for the synthesis of polybasic lipidated peptides in solution or on a solid support. In both cases an *S*-prenylated cysteine methyl ester is introduced as a building block and Fmoc and Aloc urethanes are employed for protection of the *N* terminus and the lysine side-chain amino groups, respectively. These methods give rapid access to the desired deprotected peptides in pure form and thereby open up new opportunities for future biological experiments to address, for instance, selective membrane targeting by Ras, Rho and related lipidated proteins.

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[1] D. Kadereit, J. Kuhlmann, H. Waldmann, *ChemBioChem* **2000**, *1*, 144 – 169; M. Völkert, M. Wagner, C. Peters, H. Waldmann, *Biol. Chem.* **2001**, *382*, 1133 – 1145.
[2] K. Kuhn, D. J. Owen, B. Bader, A. Wittinghofer, J. Kuhlmann, H. Waldmann, *J. Am. Chem. Soc.* **2001**, *123*, 1023 – 1035; E. Nägele, M. Scheelhaas, N. Kuder, H. Waldmann, *J. Am. Chem. Soc.* **1998**, *120*, 6889 – 6902.
[3] D. Kadereit, H. Waldmann, *ChemBioChem* **2000**, 3, 200 – 203.
[4] For solid-phase approaches, see: a) P. Mayer-Fligge, J. Volz, U. Krüger, E. Sturm, W. Gernandt, K. P. Schäfer, M. Przybylski, *J. Pept. Sci.* **1998**, *4*, 355 – 363; b) B. Denis, E. Trifilieff, *J. Pept. Sci.* **2000**, 6, 372 – 377; c) S. P. Creaser, B. R. Peterson, *J. Am. Chem. Soc.* **2002**, *124*, 2444 – 2445; d) B. Ludolph, F. Eisele, H. Waldmann, *J. Am. Chem. Soc.* **2002**, *124*, 5954 – 5955; e) E. K. Dolence, J. M. Dolence, C. D. Poulter, *Bioconjugate Chem.* **2001**, *12*, 35 – 43.
[5] a) A. Welmann, M. M. Burger, J. Hagmann, *Oncogene* **2000**, *19*, 4582 – 4591; b) M. O. Roy, R. Leventis, J. R. Silvius, *Biochemistry* **2000**, *39*, 8298 – 8307; c) A. Apolloni, I. A. Prior, M. Lindsay, R. G. Parton, J. F. Hancock, *Mol. Cell Biol.* **2000**, *20*, 2475 – 2487; d) E. Choy, V. K. Chiu, J. Silletti, M. Feoktistov, T. Morimoto, D. Michaelson, I. E. Ivanov, M. R. Philips, *Cell* **1999**, *98*, 69 – 80.
[6] S. M. Sebti, A. D. Hamilton, *Oncogene* **2000**, *19*, 6584 – 6593; S. M. Sebti, A. D. Hamilton, *Expert Opin. Invest. Drugs* **2000**, *9*, 2767 – 2782.
[7] F. Ghomashchi, X. Zhang, L. Liu, M. H. Gelb, *Biochemistry* **1995**, *34*, 11 910 – 11 918.
[8] M. Gelb, personal communication.
[9] R. Leventis, J. R. Silvius, *Biochemistry* **1998**, *37*, 7640 – 7648.
[10] T. Schmittberger, H. Waldmann, *Bioorg. Med. Chem.* **1999**, *7*, 749 – 762.
[11] E. J. Corey, C. U. Kim, M. Takeda, *Tetrahedron Lett.* **1972**, 4339 – 4342.
[12] S. Rio, J.-M. Beau, J.-C. Jacquinet, *Carbohydr. Res.* **1991**, *219*, 71 – 90.
[13] Senn Chemicals.
[14] M. Dessolin, M.-G. Guillerez, N. Thieriet, F. Guibé, A. Loffet, *Tetrahedron Lett.* **1995**, 5741 – 5744.
[15] Novabiochem.
[16] D. Kadereit, P. Deck, I. Heinemann, H. Waldmann, *Chem. Eur. J.* **2001**, *7*, 1184 – 1193.

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Binding and Docking of Synthetic Heterotrimeric Collagen Type IV Peptides with $\alpha1\beta1$ Integrin

Barbara Saccà,[a] Eva-Kathrin Sinner,[a] Jens Kaiser,[a] Christoph Lübken,[b] Johannes A. Eble,[b] and Luis Moroder*[a]

KEYWORDS:

binding affinity · cell adhesion · collagen · integrins · structure elucidation

Collagen type IV, whose major and ubiquitous form consists of one $\alpha2$ and two $\alpha1$ chains,[1, 2] forms a network that determines the biomechanical stability and macromolecular organization of the basement membrane and provides a scaffold into which other constituents of the tissue are incorporated[3]. This collagen

[a] *Prof. Dr. L. Moroder, Dr. B. Saccà, Dr. E.-K. Sinner, Dr. J. Kaiser*
Max-Planck-Institut für Biochemie
Am Klopferspitz 18a, 82152 Martinsried (Germany)
Fax: (+49) 89-8578-2847
E-mail: moroder@biochem.mpg.de
[b] *Dipl.-Chem. C. Lübken, Prof. Dr. J. A. Eble*
Institut für Physiologische Chemie und Pathobiochemie
Universitätsklinikum Münster
Waldeyerstrasse 15, 48149 Münster (Germany)

also plays an important role in cell-adhesion processes that are specifically mediated by cell-surface receptors, that is, the integrins.[4] These molecules are transmembrane heterodimeric glycoproteins composed of noncovalently associated α and β subunits. Among the various known integrins, $\alpha1\beta1$ and $\alpha2\beta1$ were identified as specific collagen receptors by a combination of different approaches.[5] Other integrin ligands such as fibronectin or fibrinogen are recognized by contiguous adhesion motifs that consist of an aspartic/glutamic acid and frequently an additional arginine residue (RGD motif),[6] whereas collagen binding to integrins often depends critically upon the native triple-helical fold of the collagen molecule.[7] Correspondingly, identification of the major cell-binding site within collagen type IV proved to be difficult, but the site was finally located in the disulfide-crosslinked trimeric 434 – 516 fragment (F4 subfragment of fragment CB3(IV))[2] by cyanogen bromide (CB) fragmentation followed by trypsin digestion. The essential amino acids of the $\alpha1\beta1$ recognition site were found to be Arg461 of the $\alpha2$(IV) chain and Asp461 of the $\alpha1$(IV) chain.[8] Such an adhesion motif, provided by a defined display of the residues on different chains, is expected to depend not only on the triple-helical fold, but also decisively upon the stagger of the single chains within the triple helix. Although fluorescence resonance energy transfer experiments performed on a related fragment of native collagen type IV (F2 subfragment of CB3(IV)) supported an $\alpha2\alpha1\alpha1'$ chain register,[9] the main scope of the present study was to structurally characterize the noncontiguous $\alpha1\beta1$ integrin binding epitope.

The two heterotrimeric collagen peptides shown in Figure 1 were synthesized in order to mimic the integrin adhesion site of collagen type IV.[10] These contain the sequence segments at residues 457 – 468 of the $\alpha1$ and $\alpha2$ chains of collagen type IV and were assembled into the two most plausible $\alpha1\alpha2\alpha1'$ and $\alpha2\alpha1\alpha1'$ registers by the use of an artificial cystine knot, as previously applied by us in the synthesis of collagen heterotrimers capable of mimicking the collagenase cleavage site of collagen type I.[11] It was assumed that a stable triple helix represents the essential prerequisite for binding of the adhesion epitope to its receptor molecule and the collagen sequences 457 – 468 were extended C- and N-terminally with two and three Gly-Pro-Hyp (GPO) repeats, respectively. Indeed, both heterotrimers A and B assume a triple-helical fold but these differ, however, in thermal stability (melting temperature, $T_m = 42\,^{\circ}C$ for trimer A and 30 °C for trimer B), a fact that indicates a surprisingly strong effect of the chain stagger on the stability of the collagen-like fold.[10]

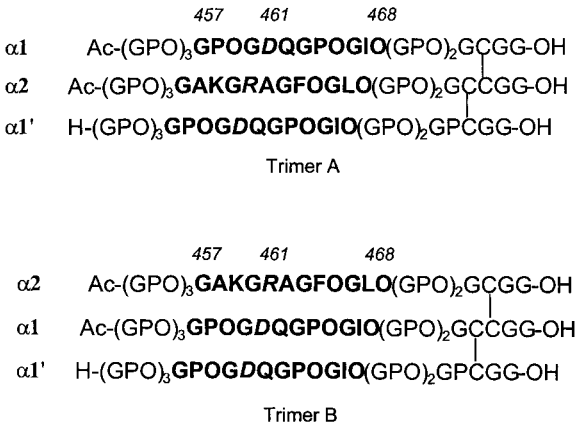

Figure 1. *Synthetic heterotrimeric collagen peptides A and B in the $\alpha1\alpha2\alpha1'$ and $\alpha2\alpha1\alpha1'$ chain registers. The $\alpha1\beta1$ integrin adhesion epitope is represented in bold by the sequence fragments 457 – 468 of the $\alpha1$ and $\alpha2$ chains of collagen type IV. The nonsequential RGD binding motif is depicted in bold italics and corresponds to the amino acid residues in position 461. Ac = acetyl.*

Analysis of the affinity of the synthetic heterotrimers A and B for the $\alpha1\beta1$ integrin was carried out by using both a commercial sample of purified human $\alpha1\beta1$ integrin (VLA-1) and a construct that consisted of the extracellular portions of the $\alpha1$ and $\beta1$ subunits heterodimerized by a Fos/Jun transcription factors. The ability of the latter integrin construct to recognize and bind collagen type IV was assessed in separate experiments that will be reported elsewhere. Binding of the heterotrimeric collagen peptides to human $\alpha1\beta1$ integrin was monitored by surface plasmon fluorescence spectroscopy (SPFS) upon fluorescence labeling of the ligand with Cy5 dye. This assay system was validated by reversible complexation of fragment CB3(IV) of native collagen type IV to the membrane-embedded integrin, which was coated on a gold surface. Dissociation of the complex by addition of ethylenediaminetetraacetic acid (EDTA) was found to be quantitative within 15 h, as reported in Figure 2 (left panel). In this system the trimer A binds to the integrin receptor with a slightly lower affinity than the trimer B (Figure 2, right panel). A quantitative evaluation of the binding affinities was obtained by isothermal titration calorimetry, for which the water-soluble integrin construct was used. As reported in Table 1, K_D values of 25 and 20 μM were extracted from the titration curves of trimers A and B, respectively. Thus, trimer A, with its $\alpha1\alpha2\alpha1'$ register, again exhibits a lower affinity than trimer B, which has a $\alpha2\alpha1\alpha1'$ chain alignment.

The X-ray structure of the molecular complex of the $\alpha2\beta1$ integrin I domain and a triple-helical homotrimeric collagen peptide that contains the Gly-Phe-Hyp-Gly-Glu-Arg binding motif of collagen type I[12] has clearly confirmed the unique mode of interaction between ligand and receptor. The Glu residue of the middle strand of the homotrimer coordinates the metal ion of the metal-ion-dependent adhesion site (MIDAS) motif whilst the adjacent arginine side chain forms a salt bridge to Asp219 in loop 2 of the integrin. This Asp219 residue in the $\alpha2$ I domain is replaced by an arginine in the $\alpha1$ integrin I domain, which allows better interaction with collagen type IV.[13] In order to try to account for this observation and to explain the different affinities of trimers A and B for $\alpha1\beta1$ integrin, modeling experiments were performed by using the X-ray structure of the I domain of the $\alpha1$ subunit.[14] The results are shown in Figure 3. Both heterotrimers contain the crucial Asp461 residue in the tailing chain, which binds to the divalent cation to provide the sixth coordinating ligand of the octahedral metal complex. In heterotrimer A (left panel) this coordination seems to be the only stabilizing interaction. Conversely, the different alignment of the chains in trimer B (right panel) allows for two additional favorable interactions with the residues of the $\alpha1$ integrin

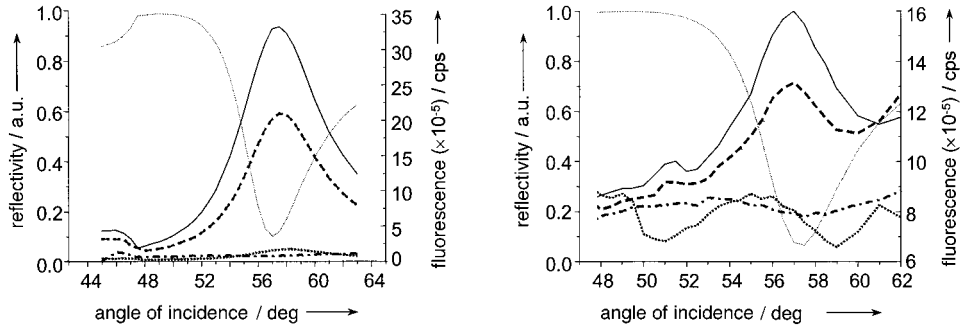

Figure 2. *SPFS binding assays of natural and synthetic collagenous peptides (fluorescence-labeled with Cy5 dye) with human $\alpha1\beta1$ integrin embedded into a lipid bilayer membrane system coated on a gold surface. Left panel: fluorescence signal upon formation of a complex between the CB3(IV) fragment of natural collagen type IV and the $\alpha1\beta1$ integrin (——) and upon dissociation of the complex 3 h (– – –) and 15 h (••••) after addition of EDTA, compared to the background fluorescence signal (•–•–); the angular dependence of reflectivity is also shown as a thin dotted line. Right panel: fluorescence signal upon binding of the monomer (••••), heterotrimer A (– – –), and heterotrimer B (——) to the integrin compared to the background fluorescence signal (•–•–); the angular dependence of reflectivity is also shown as a thin dotted line.*

Table 1. *Thermodynamic parameters of binding of the heterotrimers A and B to $\alpha1\beta1$ integrin.*

collagenous peptides	ΔH_{bind}[a] [kJ mol^{-1}]	K_D[a] [μM]	ΔS_{bind}[b] [J mol^{-1} K^{-1}]	ΔG_{bind}[c] [kJ mol^{-1}]
heterotrimer A	−20.9	25	12.6	−24.4
heterotrimer B	−18.8	20	22.0	−24.9

[a] Association enthalpies (ΔH_{bind}) and dissociation constants (K_D) were determined by isothermal titration calorimetry measurements at 4 °C in Tris·HCl (50 mM, pH 7.4), NaCl (150 mM), $MgCl_2 \cdot 6H_2O$ (2 mM), and $MnCl_2 \cdot 2H_2O$ (1 mM). [b] Entropy contributions (ΔS_{bind}) were determined from the standard equation that applies at equilibrium, $\Delta G_{bind} = \Delta H_{bind} - T\Delta S_{bind}$. [c] Free energies of binding (ΔG_{bind}) were calculated from the binding constants K_{bind} as $-RT\ln K_{bind}$.

binding region. The Asp461 residue in the middle chain of the trimer forms a salt bridge to Arg108 of the receptor (which corresponds to Asp219 of $\alpha2\beta1$ integrin) and Lys459 of the leading chain is salt bridged to Glu145 of the $\alpha1$ subunit. Since salt bridges contribute significantly to binding interactions between protein molecules,[15] this different pattern of salt bridges may well be responsible for the higher binding affinity of heterotrimer B when compared to heterotrimer A. Moreover, as the triple-helical fold of trimer B is less stable (see above), optimal fitting to the binding site may occur more easily.

Attempts to dock both collagenous heterotrimers into the I domain of the $\alpha2\beta1$ integrin failed since the Asp461 residue cannot coordinate the metal ion of the MIDAS region. Within the limits of such modeling experiments, this observation agrees with the higher binding affinity of collagen type IV for $\alpha1\beta1$ integrin rather than $\alpha2\beta1$.[13] In conclusion, all the experimental data strongly suggest that the $\alpha2\alpha1\alpha1'$ alignment of the chains represents the natural register of collagen type IV.

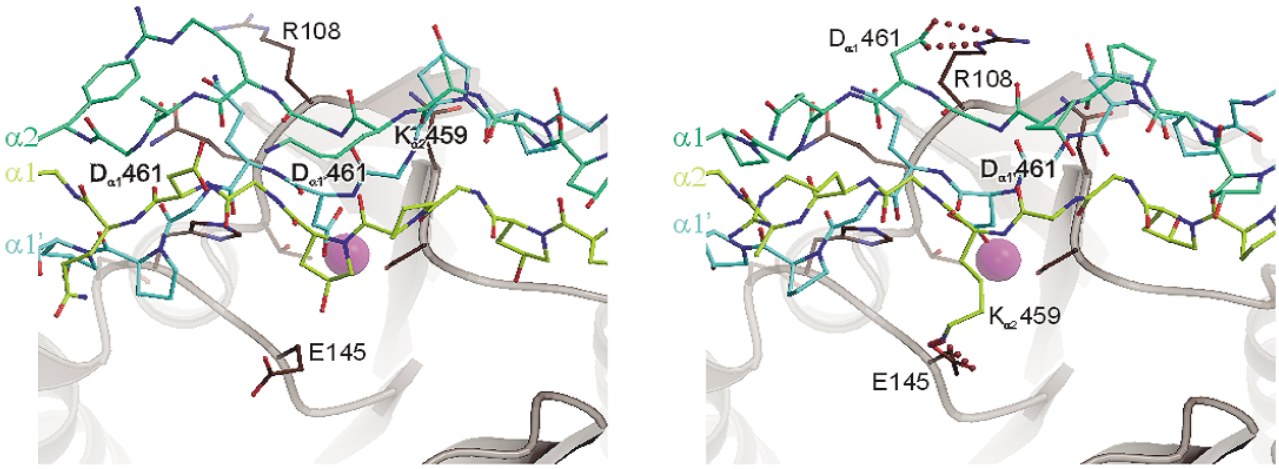

Figure 3. *View of heterotrimers A (left) and B (right) docked to the MIDAS-centered trench of the I domain of $\alpha1\beta1$ human integrin. The backbone of the MIDAS region is shown in grey ribbons. The three strands of the collagenous peptides are represented as colored sticks with the leading strand in light green, the middle strand in darker green, and the tailing strand in turquoise, and thus show the $\alpha1\alpha2\alpha1'$ and the $\alpha2\alpha1\alpha1'$ alignments of the heterotrimers A and B, respectively. The metal ion is shown as a magenta ball and hydrogen bonds are depicted as red dotted lines. In both heterotrimers the principal interaction involves coordination of the Asp461 of the $\alpha1'$ chain (turquoise) to the metal ion. Additional favorable interactions were found in heterotrimer B (right), where Lys459 of the $\alpha2$ leading strand forms a salt bridge to Glu145, and Asp461 of the $\alpha1$ middle chain forms a salt bridge to Arg108. Conversely, the arrangement of the chains in heterotrimer A does not allow stabilizing interactions with residues of the $\alpha1$ integrin I domain (compare with Figure 1 in the paper by Emsley et al.[12]). Dark blue = nitrogen; red = oxygen.*

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Experimental Section

Surface plasmon resonance fluorescence spectroscopy (SPFS): The SPFS binding experiments were carried out with a self-assembled set-up.[16] The CB3(IV) fragment of collagen type IV and the two synthetic heterotrimers were fluorescence-labeled with Cy5 dye (Amersham Pharmacia, Uppsala, Sweden) and the purified human $\alpha1\beta1$ integrin (Chemicon Inc., Temecula, Ca) was embedded into a dimyristoyl phosphatidylethanolamine/phosphatidylcholine bilayer coated onto a gold surface through use of a hydrophilic laminin peptide layer. The binding experiments were performed at 25 °C in tris(hydroxymethyl)aminomethane (Tris)·HCl (50 mM, pH 7.4), NaCl (150 mM), $MgCl_2 \cdot 6H_2O$ (2 mM), and $MnCl_2 \cdot 2H_2O$ (1 mM).

Isothermal titration calorimetry (ITC): ITC measurements were performed at 4 °C on MicroCal VP-ITC equipment by titration of a solution (0.5 μM) of $\alpha1\beta1$ integrin Fos/Jun construct in Tris·HCl (50 mM, pH 7.4), NaCl (150 mM), $MgCl_2 \cdot 6H_2O$ (2 mM), and $MnCl_2 \cdot 2H_2O$ (1 mM) with a solution of each heterotrimer (20 μM) in the same buffer. The data were fitted by using a nonlinear least squares Marquardt algorithm with three independent variables, K_{bind}, ΔH_{bind}, and the stoichiometry N (here fixed as 1);[17, 18] a single set of identical sites was used as the fitting model.

Modeling Experiments: Complex models were created by homology modeling with the program MAIN.[19] Energy minimization was performed starting from the X-ray structure 1QC5[14] of the $\alpha1\beta1$ I domain and a model for the heterotrimers based on the structure of the homotrimer in 1DZI.[12] In addition to the atomic force field, constraints were used to tether homologous Cα positions of the complex model to the 1DZI structure.

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[1] B. Trueb, B. Grobli, M. Spiess, B. F. Odermatt, K. H. Winterhalter, *J. Biol. Chem.* **1982**, *257*, 5239 – 5245.
[2] P. Vandenberg, A. Kern, A. Ries, L. Luckenbill-Edds, K. Mann, K. Kühn, *J. Cell Biol.* **1991**, *113*, 1475 – 1483.
[3] P. D. Yurchenco, J. C. Schittny, *FASEB J.* **1990**, *4*, 1577 – 1590.
[4] R. O. Hynes, *Cell* **1987**, *48*, 549 – 554.
[5] D. S. Tuckwell, M. J. Humphries, *Crit. Rev. Oncol. Hematol.* **1993**, *15*, 149 – 171.
[6] M. D. Pierschbacher, E. Ruoslahti, *Nature* **1984**, *309*, 30 – 33.
[7] K. Kühn, J. Eble, *Trends Cell Biol.* **1994**, *4*, 256 – 261.
[8] J. A. Eble, R. Golbik, K. Mann, K. Kühn, *EMBO J.* **1993**, *12*, 4795 – 4802.
[9] R. Golbik, J. A. Eble, A. Ries, K. Kühn, *J. Mol. Biol.* **2000**, *297*, 501 – 509.
[10] B. Saccà, L. Moroder, *J. Pept. Sci.* **2002**, *8*, 192 – 204.
[11] J. Ottl, D. Gabriel, G. Murphy, V. Knäuper, Y. Tominaga, H. Nagase, M. Kröger, H. Tschesche, W. Bode, L. Moroder, *Chem. Biol.* **2000**, *7*, 119 – 132.
[12] J. Emsley, C. G. Knight, R. W. Farndale, M. J. Barnes, R. C. Liddington, *Cell* **2000**, *101*, 47 – 56.
[13] C. G. Knight, L. F. Morton, A. R. Peachey, D. S. Tuckwell, R. W. Farndale, M. J. Barnes, *J. Biol. Chem.* **2000**, *275*, 35 – 40.
[14] R. L. Rich, C. C. S. Deivanayagam, R. T. Owens, M. Carson, A. Hook, D. Moore, V. W. C. Yang, S. V. L. Narayana, M. Hook, *J. Biol. Chem.* **1999**, *274*, 24906 – 24913.
[15] S. Kumar, B. Ma, C. J. Tsai, R. Nussinov, *Proteins* **2000**, *38*, 368 – 383.
[16] E. K. Schmidt, T. Liebermann, M. Kreiter, A. Jonczyk, R. Naumann, A. Offenhausser, E. Neumann, A. Kukol, A. Maelicke, W. Knoll, *Biosensors & Bioelectronics* **1998**, *13*, 585 – 591.
[17] I. Jelesarov, H. R. Bosshard, *J. Mol. Recognit.* **1999**, *12*, 3 – 18.
[18] J. E. Ladbury, B. Z. Chowdhry, *Chem. Biol.* **1996**, *3*, 791 – 801.
[19] D. Turk, Thesis, Technische Universität München, **1992**.

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Computational Studies of Subtilisin-Catalyzed Transesterification of Sucrose: Importance of Entropic Effects.

Gloria Fuentes,[a, b] M. Ángeles Cruces,[b] Francisco J. Plou,[b] Antonio Ballesteros,[b] and Chandra S. Verma*[a]

KEYWORDS:

carbohydrates · computer studies · entropy · enzymes · regioselectivity

Introduction

Regioselective acylation of sugars at their hydroxy groups is currently of great importance for the food, chemical, and pharmaceutical industries.[1] Enzymes are very useful for catalysis of these processes owing to their exquisite chemo-, stereo-, and regioselectivity,[2] and subtilisins are among the most researched. One of these specific transformations is the transesterification of sucrose with fatty acid esters with a high selectivity for the 1′-position (Figure 1; experimentally, 80 – 90% of the products are the 1′-product while 2 – 5% are minor species that are largely monoesters whose acylation positions could not be assigned; there is no available thermodynamic data on these reactions).[3]

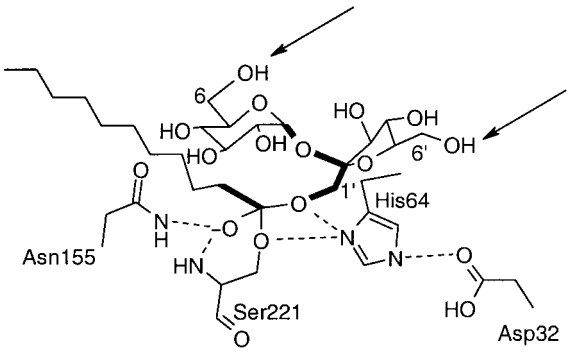

Figure 1. *Schematic representation of the subtilisin active site (catalytic triad and oxyanion hole) and the centres of reactions (the 6OH, 6′OH, and 1′OH sites) in the sucrose moiety. The torsions across which conformational sampling was carried out are indicated by dark lines.*

[a] *Dr. C. S. Verma, G. Fuentes*
Structural Biology Laboratory
Department of Chemistry
University of York, York, YO10 5DD (UK)
Fax: (+44) 1904-410519
E-mail: chandra@ysbl.york.ac.uk

[b] *G. Fuentes, Dr. M. Á. Cruces, Dr. F. J. Plou, Prof. A. Ballesteros*
Departamento de Biocatálisis
Instituto de Catálisis
CSIC, Cantoblanco, 28049 Madrid (Spain)