Penicillin G Amidase-Catalysed Hydrolysis of Phenylacetic Hydrazides on a Solid Phase: A New Route to Enzyme-Cleavable Linkers

Alessandra Basso,[a] Cynthia Ebert,[a] Lucia Gardossi,[a,*] Paolo Linda,[a] Thao Tran Phuong,[b] Mingzhao Zhu,[b] Ludger Wessjohann[b,*]

[a] Laboratory of Applied and Computational Biocatalysis, Dipartimento di Scienze Farmaceutiche, Università degli Studi, Piazzale Europa 1, 34127, Trieste, Italy
Phone: (+39)-040-558-3110, Fax: (+39)-040-52572, e-mail: gardossi@units.it
[b] Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120 Halle (Saale), Germany
Phone: (+49)-345-5582-1301, Fax: (+49)-345-5582-1309, e-mail: wessjohann@ipb-halle.de

Received: January 23, 2005; Accepted: March 29, 2005

Abstract: A novel catalytic property of penicillin G amidase (PGA) is described. Unexpectedly, the enzyme can hydrolyse hydrazide bonds with good efficiency, and in solution the enzyme shows a selectivity that is similar to phenylacetamides. The hydrolysis of phenylacetic hydrazides releases hydrazine, but no inhibition due to the formation of such reactive compounds was observed. This novel catalytic property was assayed also on a solid phase as a pioneering route for the design of enzyme-cleavable linkers and masked scavengers for ketones. On a solid phase a phenylacetic hydrazide compound was chemically synthesised on $PEGA_{1900}$ and $PEGA^+$ (two co-polymers of acrylamide and ethylene glycol) and the efficiency of PGA in the release of phenylacetic acid depended on the diffusion of the protein inside the polymer. On $PEGA^+$ the enzyme, as previously described, shows a good diffusion due to an improved electrostatic interaction with PGA thus achieving good hydrolytic conversions.

Keywords: hydrazine; hydrolysis; penicillin G amidase; phenylacetic hydrazide; solid phase biocatalysis

The success of solid phase synthesis lies on the choice of a suitable support and an appropriate linker. Solid phase synthesis involves the use of linkers between the compounds and the solid supports which must be stable during the reactions. These linkers have to be cleavable as desired, usually at the end of the synthetic sequence, with high selectivity and in good yield, without affecting the structures of the products that are released from the polymeric support. In this way molecules can be assembled, modified and finally released through the cleavage of the linker.

Previously, linkers have mostly been cleaved by classical chemical methods, for instance, using strong acids.[1] Such conditions often restrict the application of linkers. Acid-sensitive linkers are not suitable for acid-labile compounds, such as carbohydrates. Specific linkers have, therefore, been developed for acid-labile compounds, such as silyl ether linkages, thioether linkages and ester linkages. However, they have the disadvantage that they are quite labile to common chemical reagents. Also, almost all linkers available are unsuitable for multifunctional compounds such as complex natural products with sensitivity to several orthogonal cleavage methods.

In organic synthesis enzymatic methods in many cases have opened up advantageous alternatives to such classical chemical techniques since enzyme-catalysed transformations typically proceed under very mild conditions (pH and temperature) and with pronounced chemo-, regio- and stereoselectivity.[2]

In particular, enzymatic transformations have enabled the establishment of alternative protecting group techniques. Therefore enzymatic transformations that may be employed for the removal of protecting groups in solution, in principle, also may open up alternative opportunities to release compounds from polymeric supports.

To this extent, penicillin G amidase, which is highly specific for phenylacetic groups, is an enzyme of first choice.[3] During the last years much effort has been made for the design of penicillin G amidase-cleavable linkers.[4]

Some years ago, Waldmann proposed a phenyl hydrazide as an enzyme-labile protecting group.[5] Using a tyrosinase, it was possible in solution to convert the chemically stable hydrazide precursor into a labile intermediate, thus releasing the free carboxylic acid. In other examples, amidases from different strains of *Rhodococcus* were used to synthesise hydrazides.[6]

Our idea was to create a linker where the high nucleophilicity of the reactive hydrazine is masked with a phenylacetic group. After the cleavage by penicillin G ami-

 DOI: 10.1002/adsc.200505038

dase the free, released hydrazine could react and release a target compound that can also be located far from this enzymatic site of cleavage. However, the design of such an enzyme-cleavable linker would be totally useless if the enzyme is not able to cleave hydrazide or hydrazone bonds and no examples of such selectivity have been reported for penicillin G amidase so far.

Here we report the first example of penicillin G amidase selectivity towards hydrazide bonds both in solution and on a solid phase. First attempts were focused on the hydrolysis of commercial phenylacetic hydrazide in solution. PGA catalysed the hydrolysis of phenylacetic hydrazide with an initial rate of hydrolysis that was only seven times lower than that of the corresponding phenylacetamide. The hydrolytic reaction was complete within 2 h. The hydrolysis of phenylacetic hydrazide results in the formation of the very reactive hydrazine that, in principle, could react with carbonyl groups of the enzyme causing damage.[7] Thus, the same hydrolytic reaction was assayed also in the presence of acetone in order to capture the released hydrazine through formation of the corresponding hydrazone (Scheme 1). Our results showed no measurable effect of the released hydrazine on the enzyme activity since the initial rates did not change upon addition of acetone. Hydrolytic reactions went to completion regardless of the presence of acetone.

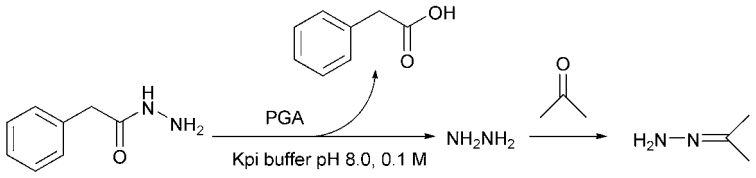
In both cases, after the complete hydrolysis of phenylacetic hydrazide, fresh substrate was added and initial rates measured. No difference was observed, thus indicating that the enzymatic activity was not affected by the released hydrazine in the range of the reaction time considered. However, it must be underlined that the enzyme might undergo covalent modifications whose effect would become apparent only after a much longer reaction time.

Starting from these encouraging results, the hydrolysis of phenylacetic hydrazides was assayed on solid phase.

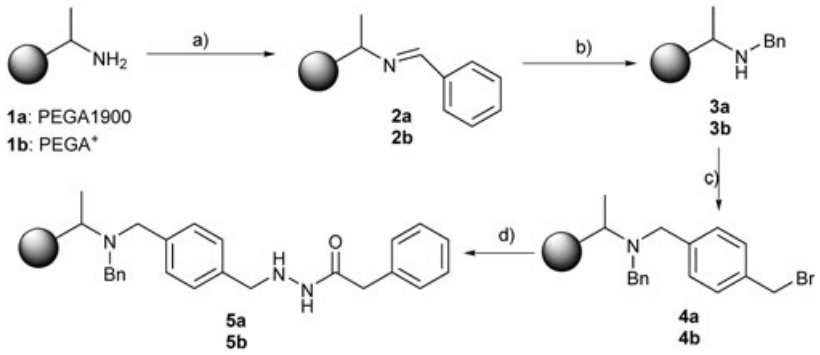
Recently, the use of PEGA resins (co-polymers of ethylene glycol and acrylamide) functionalised with hydrazides, as new scavenger for the reversible linking of aldehydes has been reported.[8] The same synthetic procedure was used for the synthesis of compound **5** on $PEGA_{1900}$ (**5a**) and $PEGA^+$ (**5b**) (Scheme 2). $PEGA^+$, produced by Polymer Laboratories by introduction of charged monomers in the $PEGA_{1900}$ structure was demonstrated to improve PGA accessibility on the solid support due to electrostatic interactions.[9,10] Penicillin G amidase that is negatively charged at pH 8.0 is attracted by the positively charged polymer so that conversions are improved.

Benzaldehyde was successfully coupled on $PEGA_{1900}$ and $PEGA^+$ leading to the imine compounds **2a, b** that, after reduction in the presence of sodium triacetoxyborohydride to give **3a, b**, were reacted with 1,4-di-(bromomethyl)benzene to give **4a, b**. The final hydrazide **5a, b** was prepared by reaction with phenylacetic hydrazide. All the initial amino groups were acylated, as demonstrated by determination of the final loading.

PGA-catalysed hydrolysis of compounds **5a, b** was then assayed using hydrolytic procedures already reported.[9,10] Enzymatic hydrolysis on $PEGA_{1900}$ (**5a**)



Scheme 1. Formation of hydrazine after hydrolysis by PGA and reaction with acetone with formation of hydrazone.



Scheme 2. a) Benzaldehyde, $HC(OMe)_3$, room temperature, 24 hours; b) $NaB(OAc)_3H$, MeOH, 1% AcOH (v/v), room temperature, 48 hours; c) 1,4-di(bromomethyl)benzene, DIPEA, DMF, room temperature, 48 hours; (d) phenylacetic hydrazide, DMF, room temperature, 48 hours.

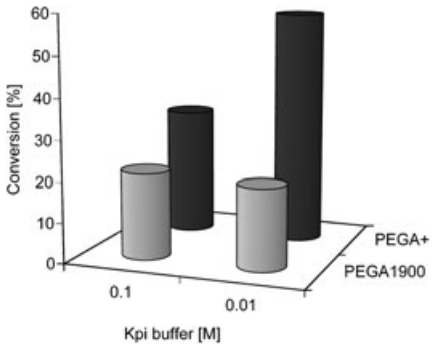


Figure 1. Comparison of the influence of polymer charge and buffer concentration on the cleavage of polymer bound phenylacetic hydrazide.

and PEGA$^+$ (**5b**), in 0.1 M Kpi buffer, lead to the formation of 0.033 and 0.063 mmol/g dry of phenylacetic acid, corresponding to conversions of 22% and 32%, respectively (Figure 1). Conversions were determined at the equilibrium, after 24 h reaction: the equilibrium position was confirmed by the addition of fresh enzyme that caused no additional observed hydrolysis. As in the case of hydrolysis of phenylacetic hydrazide in solution, it was also verified that the release of phenylacetic acid did not cause any shift in the pH of the medium, even when reactions were performed at 0.01 M buffer concentration.

As demonstrated before, the use of positively charged polymers (PEGA$^+$) in diluted buffer can improve the accessibility of penicillin G amidase.[9] Indeed, when the hydrolysis of PEGA$^+$ derived compound **5b** was performed in 0.01 M Kpi buffer, the release of phenylacetic acid increased to almost 60%, whereas with PEGA$_{1900}$ the conversion was unacceptably low (20%).

This novel catalytic property of PGA offers undoubted potential to organic chemists: highly reactive hydrazines can be masked by a stable phenylacetic hydrazide bond that can be selectively cleaved through enzymatic hydrolysis. We are currently investigating the steric requirements of different hydrazide substrates for PGA with the aid of computational methods and an experimental approach. Information will be exploited for the rational design of specific linker structures that can be cleaved by PGA.

Experimental Section

Enzymatic Hydrolysis

0.1 mmol of phenylacetamide or phenylacetic hydrazide were dissolved in 2 mL of KH_2PO_4/K_2HPO_4, Kpi buffer (0.1 M, pH 8.0). 1 mL of a penicillin G amidase solution (prepared by dissolving 5 mg of native enzyme in 10 mL of Kpi buffer, 14 U/mg after lyophilisation of a solution from Fluka) was added to start the reaction. Reaction components were mixed at 30 °C, 250 rpm. Initial rates were calculated by withdrawing samples and analysing with RP-HPLC (60% H_2O, 40% MeCN, 0.1% TFA in both phases). Reactions with acetone were performed by adding 0.2 mmol of acetone (or acetophenone). v_0 phenylacetamide: 7.9 μmol/min; v_0 phenylacetic hydrazide: 1.2 μmol/min. Blanks were run of all experiments and the results were negative.

Hydrolyses of compounds **5a, b** were performed as described before.[9,10]

Synthesis of Compound 5a (PEGA$_{1900}$) and 5b (PEGA$^+$)

The synthesis is similar to that described in detail in ref.[8] Thus, PEGA$_{1900}$ and PEGA$^+$ were reacted with benzaldehyde in the presence of trimethyl orthoformate for 24 h and room temperature to give **2a, b**. Compound **2a, b** was reduced with sodium triacetoxyborohydride in the presence of methanol/acetic acid (1% v/v) at room temperature for 48 h. After washing the resins **3a, b**, they were reacted with di(bromomethyl)benzene and diisopropylethylamine in DMF for 48 h at room temperature. Products **5a, b** were obtained by reacting **4a, b** with phenylacetohydrazide in DMF at room temperature for 48 h.

Loading Determination

The corresponding hydrazide of compound **4a** (1 g of wet resin corresponding to about 0.1 g dry) was reacted with FMOC-chloride (9-fluorenylmethyl chloroformate; 20 equivs., 0.3 mmol), DIPEA (diisopropylethylamine, 20 equivs.) in 10 mL dry DMF. The reaction mixture was stirred for 30 minutes, then a second coupling occurred. The loading was determined by cleavage of FMOC group in the presence of piperidine[9] taking into account the double acylation on nitrogens of the hydrazide group. PEGA$_{1900}$: 0.15 mmol/g, PEGA$^+$: 0.2 mmol/g.

Acknowledgements

The authors gratefully acknowledge financial support from the EU (COMBIOCAT project). Thanks are due to Polymer Laboratories for providing PEGA resin as part of this project (http://www.polymerlaboratories.com).

References

[1] K. Gordon, S. Balasubramanian, *J. Chem. Technol. Biotechnol.* **1999**, *74*, 835–851.
[2] R. Reents, D. A. Jeyaraj, H. Waldmann, *Adv. Synth. Catal.* **2001**, *343*, 501–513.
[3] H. Waldmann, D. Sebastian, *Chem. Rev.* **1994**, *94*, 911–937.
[4] a) U. Grether, H. Waldmann, *Angew. Chem. Int. Ed.* **2000**, *39*, 1629–1632; b) U. Grether, H. Waldmann, *Chem. Eur. J.* **2001**, *7*, 959–971; c) G. Böhm, J. Dowden, D. C. Rice, I. Burgess, I. J. F. Pilard, B. Guilbert, A. Hax-

ton, R. C. Hunter, N. J. Turner, S. L. Flitsch, *Tetrahedron Lett.* **1998**, *39*, 3819–3822.
[5] G. H. Müller, H. Waldmann, *Tetrahedron Lett.* **1999**, *40*, 3549–3552.
[6] a) D. Fournand, A. Arnaud, P. Galzy, *J. Mol. Catal. B: Enzymatic* **1998**, *4*, 77–90; b) M. Kobayashi, M. Goda, S. Shimizu, *Biochem. Biophys. Research Commun.* **1999**, *256*, 415–418.
[7] M. See, M. A. Graziewicz, B. J. Day, W. C. Copeland, *Nucl. Acid Res.* **2002**, *30*, 2817–2824.
[8] M. Zhu, E. Ruijter, L. Wessjohann, *Org. Lett.* **2004**, *6*, 3921–3924.
[9] A. Basso, L. De Martin, L. Gardossi, G. Margetts, I. Brazendale, A. Y. Bosma, R. V. Ulijin, S. L. Flitsch, *Chem. Commun.* **2003**, 1296–1297.
[10] a) A. Basso, R. V. Ulijn, S. L. Flitsch, G. Margetts, I. Brazendale, C. Ebert, L. De Martin, P. Linda, S. Verdelli, L. Gardossi, *Tetrahedron* **2004**, *60*, 589–594; b) A. Basso, B. A. Maltman, S. L. Flitsch, G. Margetts, I. Brazendale, C. Ebert, P. Linda, S. Verdelli, L. Gardossi, *Tetrahedron* **2005**, *61*, 971–976.

